

Review

Proteomics in Forensic Analysis: Applications for Human Samples

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Abstract: Proteomics, the large-scale study of all proteins of an organism or system, is a powerful tool for studying biological systems. It can provide a holistic view of the physiological and biochemical states of given samples through identification and quantification of large numbers of peptides and proteins. In forensic science, proteomics can be used as a confirmatory and orthogonal technique for well-built genomic analyses. Proteomics is highly valuable in cases where nucleic acids are absent or degraded, such as hair and bone samples. It can be used to identify body fluids, ethnic group, gender, individual, and estimate post-mortem interval using bone, muscle, and decomposition fluid samples. Compared to genomic analysis, proteomics can provide a better global picture of a sample. It has been used in forensic science for a wide range of sample types and applications. In this review, we briefly introduce proteomic methods, including sample preparation techniques, data acquisition using liquid chromatography-tandem mass spectrometry, and data analysis using database search, spectral library search, and de novo sequencing. We also summarize recent applications in the past decade of proteomics in forensic science with a special focus on human samples, including hair, bone, body fluids, fingernail, muscle, brain, and fingerprint, and address the challenges, considerations, and future developments of forensic proteomics.

Keywords: forensics; proteomics; biomarker; human identification; serology; hair; bone



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1. Introduction

Proteomics is the study of proteomes (i.e., the total proteins of a given sample such as cultured cells, a tissue, or an organism) and their changes in response to environmental or physiological conditions [1]. Proteomics identifies proteome profiles of samples, thereby revealing the biological status of the samples as well as their regulatory or disease mechanisms. Proteomics has been widely used to study microbiology, cell and molecular biology, plant sciences, marine sciences, food sciences, cancer, and immunology [2]. The development of proteomics relies on a number of technologies and techniques, including liquid chromatography-tandem mass spectrometry (LC-MS/MS) and statistical and bioinformatics tools [3].

Proteomics is a powerful approach for studying biological systems. Recent developments in LC-MS/MS have allowed rapid analyses of peptides and proteins in samples, which is comparable to next-generation sequencing (NGS). Compared with immunological methods that require antibodies and polymerase chain reaction (PCR) using specific primers, proteomics may reduce time and overall analysis costs. It does not depend on the development of new antibodies or primers for specific proteins [4]. Proteomics enables the identification and quantification of various peptides and proteins in a single experiment with high specificity. Thus, it not only measures a large number of targets, but also provides

a holistic view of the physiological and biochemical states of given samples [2]. A proteome profile, including proteins and their abundances, provides a better global picture of a sample than static DNA information. In other words, proteomics can determine biological processes that are occurring [5].

While traditional gel-based proteomics is labor-intensive and time-consuming, the development of LC-MS/MS-based proteomics in the last two decades allows separation and analysis of thousands of peptides and proteins in several hours, which considerably increases the throughput of proteomics [6]. It also possesses some advantages as compared with the well-established immunoassays, such as higher specificity and repeatability, reduced time, labor, and long-term cost, the ability of multiplexing. Particularly, proteomics can avoid the requirement of genetically tagged proteins and specific antibodies for each protein [7]. In addition, the sample preparation workflow of proteomics can be automated using some liquid handling workstations, such as Agilent AssayMAP Bravo [8] and Biomek NXP Span-8 [9]. Most of the steps in bottom-up and top-down proteomics can be automated to increase precision, repeatability, and high-throughput performance [10]. In biological forensics, DNA is undoubtedly effective for human identification from various sample types, such as blood, skin, urine, and hair. However, in some cases, DNA might not answer all questions relating to forensics, such as the biological fluid or tissue type of a sample [4]. DNA in samples is sometimes unavailable or degraded, whereas proteins can persist and thus, can be analyzed instead [11]. Proteins themselves are also the targeted biomolecules when analyzing protein toxins, protein drugs, and hormones. Therefore, proteomics can be a confirmatory and orthogonal technique for well-built DNA sequencing, and an additional strategy to reveal other information [4]. Currently, forensic proteomics is in the early stages of development. An increasing number of studies have used proteomics in forensics, such as identification of tissue and body fluid [12–14], identification and quantification of protein toxins [15,16], human individualization [17,18], detection of protein drugs and hormones in sports [19,20], and discrimination of wild strains and laboratory-adapted strains of bacteria [21,22].

Several reviews and book chapters on forensic proteomics are available. Recently, Merkley et al. presented a standard bottom-up proteomic workflow [23]. In addition, Nicora et al. described details of sample preparation in bottom-up proteomics [24], and Cole et al. focused on proteomics sample treatment procedures applied for tissues [25]. Another review summarized some applications of proteomics in forensics, including human identification from hair samples and identification of body fluids [11]. A recent review presented some forensic proteomic applications, such as human samples (hair, bone, and body fluids), species identification, toxins, and microbial forensics [4]. Our current review aims to focus on a wide range of human samples used in forensic proteomics with updated findings from the literature. They include hair, bone, body fluids (blood, urine, semen, vaginal fluid, saliva), muscle, fingernail, muscle, brain, and fingermarks. These samples are different from each other in terms of sample amount, sample availability, and protein amount. The amount of sample collected may vary depending on the forensic contexts. Some samples may have relatively low amounts, such as hair, body fluids, tissues, and their traces in fingernail and fingermark. Protein amounts are different among samples. In sport doping, urine and blood can be used. Urine usually has low protein content as compared with that of blood; however, it is easily available. Some samples, such as bone, muscle, brain, and decomposition fluid might be only available from corpse. Human samples can be used for various forensic proteomic applications. Hair is a valuable sample for identifications of ethnic groups, gender, and individual [18,26,27]. Urine and blood are used to identify and quantify illegal peptides and small protein hormones for sport doping [28]. Some samples, such as bone, muscle, brain, cerebrospinal fluid, and decomposition fluid are used to estimate post-mortem interval (PMI) [29–33]. Fingernail and fingermark may contain traces of body fluids and tissues and thus, be used for their identification [34,35]. Table 1 summarizes some applications of proteomics in forensics using different types of human samples.

Table 1. Applications of forensic proteomics using human samples.

Sample Type	Applications
Hair	Identify ethnic groups [18,36,37] Distinguish gender [26] Distinguish individuals [27,38,39]
Bone	Estimate biological age [29,40–42] Estimate post-mortem interval (PMI) [29,30] Distinguish individuals [43]
Body fluid and tissue	Identify body fluids using biomarkers [12,14,44] Identify tissues using biomarkers [45,46]
Urine and blood	Identify and quantify illegal peptides and small protein hormones for sport doping [28,47,48]
Fingernail	Identify body fluids and tissues trapped under fingernails [34]
Muscle	Estimate mid-PMI [31]
Brain and cerebrospinal fluid	Estimate PMI [32] Identify diagnostic biomarkers for sudden infant death syndrome (SIDS) [49]
Blood	Identify biomarkers in the postmortem diagnosis of drowning [50]
Decomposition fluid	Estimate PMI [33]
Fingermark	Identify traces of body fluids [35] Determine age-markers of fingermarks [51]
Epidermal corneocyte	Distinguish individuals [52]

In this review, we present an overview of proteomics in forensic analysis, with a particular focus on applications in human samples. We introduce general proteomics methods that are applied in forensic studies, including sample preparation techniques, data acquisition using LC-MS/MS, and data analysis using database search, spectral library search, and de novo sequencing. Notably, we summarize recent applications in the past decade of forensic proteomics that use human samples for analysis, such as hair, bone, body fluids, muscle, fingernail, brain, blood, and fingermarks.

2. Proteomics Methods: Sample Preparation Techniques, Data Acquisition, and Data Analysis

Proteomics can be separated into top-down, middle-down, and bottom-up approaches. In top-down proteomics, intact proteins extracted from samples are directly separated and analyzed by LC-MS/MS. This allows the identification of proteoforms with post-translational modifications (PTMs) [53]. The disadvantages of top-down proteomics include difficulties in protein separation, protein solubility, MS analysis, and quantification [54]. The top-down approach is widely used in sports anti-doping to identify banned peptides or proteins [20]. In contrast, in bottom-up proteomics, proteins are digested into thousands of peptides by enzymes such as trypsin and LysC. The resulting peptides are then smaller and easier to analyze using LC-MS/MS. Prior to this analysis, peptide mixtures are usually separated into several fractions to reduce sample complexity [55]. In middle-down proteomics, protein digestion is also carried out, but the aim is to yield relatively larger peptides (2.5–10 kDa) compared with those in bottom-up proteomics. It requires special proteases, such as OmpT, Sap9, and IdeS [56]. Middle-down proteomics can reduce the complexity of the digests and enable the identification of proteoforms. The sequence coverage of the proteins can also be increased. [57]. Among three approaches, bottom-up proteomics is more feasible and applicable than top-down and middle-down proteomics, and has been widely applied in forensics. Thus, this section introduces and discusses the methods used in bottom-up proteomics. Methods and considerations of the top-down

and middle-down proteomics can be found in some literature reviews (e.g., [58,59] for top-down and [56] for middle-down proteomics). An overview of general methods for bottom-up proteomics is presented in Figure 1 [23]. A bottom-up proteomic study includes three main parts: sample preparation, data acquisition, and data analysis.

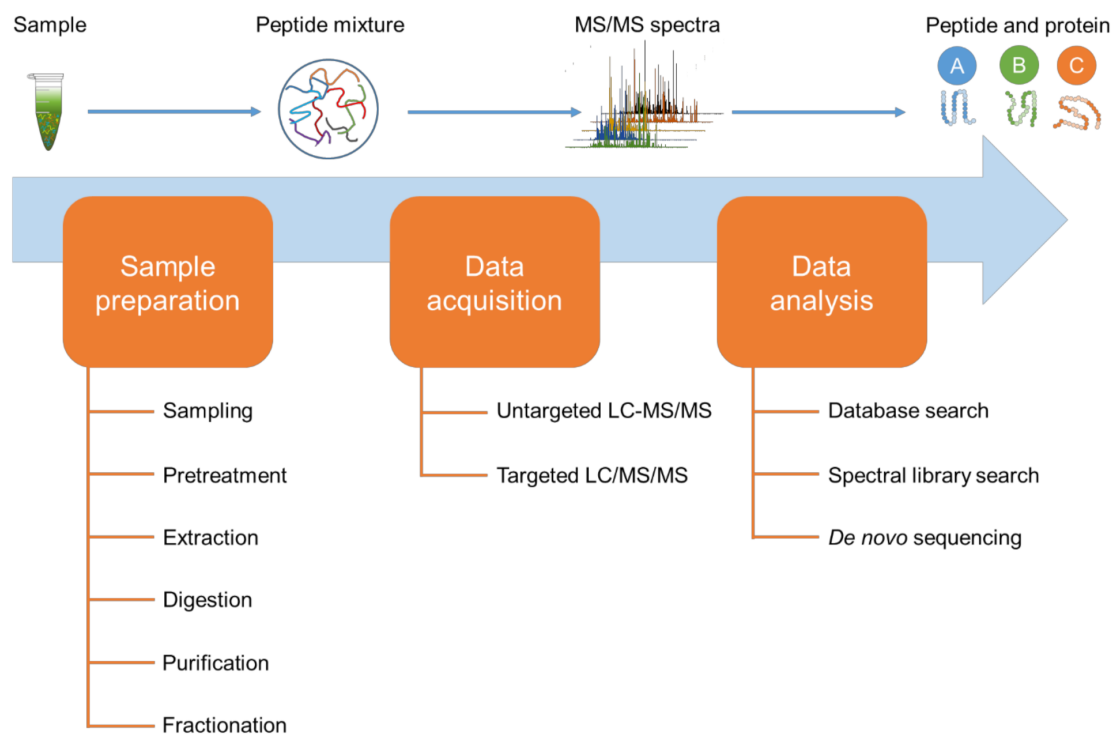


Figure 1. Overview of bottom-up proteomics.

2.1. Sample Preparation

A typical sample preparation workflow in bottom-up proteomics includes sample pretreatment, protein extraction, proteolytic digestion, and sample cleanup or peptide purification. Fractionation is sometimes carried out to reduce sample complexity and improve in-depth analysis.

2.1.1. Sampling and Sample Pretreatment

Samples can be collected from human bodies and crime scenes. Body fluids are usually collected using wide-bore pipettes or disposable hypodermic syringes with appropriate needle gauges and lengths. Antemortem nail clippings are obtained from all fingers and toes using Teflon-coated stainless steel scissors, whereas post-mortem nails are lifted from all fingers and toes. Most of the samples can be stored in appropriate plastic containers with screw caps [60]. Sample collection devices in crime scenes include wipes, dry swabs, premoistened swabs, aspirating needles, high-efficiency particulate air vacuums, and filters [61]. After collection, hair and nail samples can be stored at room temperature. Other samples should be stored in tightly-sealed containers at 4 °C for short-term storage and at −20 °C or preferably at −80 °C for long-term storage. Plasma and serum required for analysis should be separated before storage [62]. Sample pretreatment methods are selected depending on the sample type. If samples containing proteins are culture media, exosomes, or body fluids such as serum, plasma, saliva, tears, nasal fluids, urine, or aqueous humor, they do not require lysis pretreatment. In these cases, an appropriate amount of buffer is added to favor subsequent enzymatic digestion steps [63,64]. If samples are cells, a lysis step is applied to break the cell membrane. Lysis is usually performed using lysis buffer and optional sonication [65]. Probe sonication is one of the most common methods of cell disruption.

In the case of solid samples such as human tissue and bone, the samples are usually disrupted by mortar and pestle, cryogenic grinding, cryo-pulverization (e.g., using Covaris CP02 cryoPrep automated dry pulverizer), and bead beating. Bone powder can be treated with a weak acid for partial demineralization prior to digestion [66]. Soft tissues such as the brain and liver are appropriate for manual pestle homogenization or bead homogenization. In manual pestle homogenization, tissues are placed into tubes (e.g., Potter homogenizer) and manually homogenized with a pestle while the tubes are kept on ice [67]. A lysis buffer is added before the homogenization. Homogenization is completed when no large pieces of tissue are observed [68]. In bead homogenization, tissues are placed in pre-chilled tubes with tungsten carbide, stainless steel, or glass beads and subjected to high-speed shaking [69]. Hard tissues (bone and fingernails) and those with tough connective fibers are appropriate for cryo-pulverization. Typically, tissues are quickly frozen in liquid nitrogen and disrupted by force [70]. For example, the Covaris CP02 cryoPrep automated dry pulverizer allows users to control the force for disruption. It is equipped with tissue tubes (strong and flexible plastic devices developed to resist extreme temperature fluxes) to prevent sample loss. Tissues were placed in these tubes, frozen, and pulverized to powder [71]. Lysis buffer solutions were added to the tubes to collect all the samples for further steps. If this system is not available, the samples are placed in a mortar with liquid nitrogen and gently ground with a pestle. Sometimes, mortar and pestle are also used for lyophilized tissues without liquid nitrogen [72,73]. Generally, the resultant samples undergo a lysis step with lysis buffer and sonication.

In many cases, samples are bound to matrices such as cloth, cotton swab, filter surface, or rock, and thus should be solubilized in an appropriate buffer solution (e.g., triethylammonium bicarbonate (TEAB), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and ammonium bicarbonate with or without detergents). Sample-bearing materials were placed inside a tube with a buffer solution and subjected to sonication or vortexing to collect the targeted samples.

2.1.2. Protein Extraction

After proteins are solubilized in buffer solutions, they are extracted or purified by concurrent removal of contaminants. Protein precipitation is one of the most commonly used methods. In this method, proteins are precipitated by organic solvents (e.g., acetone, methanol, or ethanol) and their mixtures with acids (e.g., trichloroacetic acid) or sodium deoxycholate. Acetone is the most commonly used solvent because of its ability to dissolve nonpolar contaminants (e.g., lipids). After precipitation, the protein pellets were collected and washed with pre-chilled acetone to remove contaminants prior to proteolytic digestion [74,75]. Other methods are also used for protein purification, including chromatography, electrophoresis, dialysis, ultrafiltration, lyophilization, and crystallization [76]. When the samples are serum, plasma, or cerebrospinal fluid, immunodepletion may be required to remove the most abundant proteins [77]. For example, a human 14 multiple affinity removal system (MARS) column can remove albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha-2-macroglobulin, alpha-1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3, and transthyretin.

Prior to further sample preparation, several methods are used to determine the concentration of protein in the samples. Colorimetric dye-based methods, such as the Bradford assay, are straightforward and fast. The binding between proteins and dye results in a color change and is used to determine protein concentrations. The advantages of these methods include compatibility with most solvents, buffers, salts, reducing substances, thiols, and metal-chelating agents. These can be performed at room temperature [78]. Bicinchoninic acid (BCA) assays (biuret methods) are based on protein-copper chelation in an alkaline environment. In this reaction, Cu^{2+} is reduced to Cu^+ , which subsequently reacts with the BCA reagent to form a purple complex. The purple color can be measured at wavelengths between 550 and 570 nm. This method is compatible with most detergents and causes less protein-protein variation than the Bradford assay. However, it is not suitable for substances

that reduce copper [79]. In Lowry assays, Cu^+ is allowed to react with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid) [80]. Fluorescent dye methods are based on protein–dye binding and direct detection of the fluorescence associated with the dye. These methods are highly sensitive and applicable to samples with low protein amount. Typical examples of fluorescent dye methods are the EZQ fluorescent assay and the Qubit protein assay [81].

2.1.3. Proteolytic Digestion

Typical protein digestion methods that have been widely used are in-gel, in-solution, on-bead, on-slide tissue digestion, filter-aided sample preparation (FASP), and suspension trapping (S-Trap). In in-solution digestion, protein solutions or pellets are usually mixed with 8 M urea. Urea and thiourea can increase protein solubility and denature protein three-dimensional structures. Proteins are then reduced (e.g., with dithiothreitol or tris(2-carboxyethyl)phosphine) and alkylated (e.g., with iodoacetamide or chloroacetamide). These steps are necessary to reduce intra- and intermolecular disulfide bonds within and between proteins [82]. Subsequently, the proteins are digested with enzymes (e.g., trypsin and LysC). One of the most frequently used enzymes is sequencing-grade modified porcine trypsin. The typical trypsin: protein ratio is 1:20 to 1:50. Trypsin cleaves peptide bonds at the amino acid residues arginine and lysine. The resultant peptides had an average size of 800–2000 Da, which is the preferred mass range for MS/MS sequencing. These MS/MS spectra are information-rich and easily interpretable for peptide and protein identification [83].

A procedure of in-gel digestion is relatively similar to in-solution digestion. Briefly, proteins are separated by 1 or 2 dimensional (1D or 2D) polyacrylamide gel electrophoresis. The gel slice is then cut into small pieces (approximately 1×1 mm). These pieces are destained with 100 mM ammonium bicarbonate/acetonitrile (1:1, *v/v*) prior to optional reduction and alkylation. After tryptic digestion, peptides are usually extracted from gel pieces using 50% ACN/5% formic acid [84]. The in-gel approach is more laborious and time-consuming than in-solution digestion. It can process only a small number of protein spots at a time.

A typical example of on-bead digestion is single-pot solid-phase-enhanced sample preparation (SP3), which allows proteomic sample preparation to be carried out in a single tube with high throughput, speed, efficiency, scalability, and flexibility [85]. A complete proteomic workflow can be performed in a single SP3 tube, including protein and peptide enrichment, cleanup, digestion, chemical isotope labeling, and fractionation. In brief, after reduction and alkylation, proteins are mixed with SP3 beads for protein–bead binding. After the binding is complete, a magnetic rack is used to induce the migration of the beads to the tube wall, and unbound supernatant is removed. The proteins bound to beads are then digested using proteolytic enzymes. Subsequently, the tube is centrifuged, and a magnetic rack is used to settle the beads onto the tube wall, which allows the separation of peptides. This method has some limitations, such as difficulties in the recovery of intact proteins from the paramagnetic beads, bead clumping, and aggregation [86].

On-slide tissue digestion is a straightforward approach typically applied for formalin-fixed paraffin-embedded (FFPE) tissue section [87]. It can be used in matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS). After sections are cut from FFPE tissue blocks, they are washed with xylene to remove paraffin and then rehydrated in a series of ethanol washes. Subsequently, antigen retrieval is carried out in Tris–HCl (pH 8.0, 95 °C, 20 min). On-slide tissue digestion is conducted using spray technologies [88].

FASP consists of several ultrafiltration steps to retain proteins over ultrafiltration membranes and remove contaminants by stepwise elution. After enzymatic protein cleavage, the membranes allow separation of peptides from undigested materials [89]. In FASP, protein pellets are mixed with a buffer solution containing sodium dodecyl sulfate for solubilization, then reduced, alkylated, and added to a 30 kDa molecular weight cut-off

(MWCO) FASP spin column. Samples are centrifuged to remove waste and contaminants, whereas proteins remain on the membrane. Proteolytic enzymes (e.g., trypsin in ammonium carbonate) are added for protein digestion. The resultant peptides are collected after centrifugation [90]. Compared with in-solution digestion, FASP can be applied to samples containing detergents (e.g., sodium dodecyl sulfate) [91].

S-Trap is an alternative to FASP and in-solution digestion. This allows for high concentrations of sodium dodecyl sulfate. Another advantage of S-Trap is its short processing time [92]. In this method, the samples are mixed with sodium dodecyl sulfate and a reduction agent. After alkylation with iodoacetamide or chloroacetamide, phosphoric acid is added to a final concentration of 1.2%, and binding buffer (90% methanol, 10% 100 mM TEAB, pH 7.1) is added, as well. Due to the presence of methanol, proteins are precipitated into fine suspensions that are stabilized by sodium dodecyl sulfate. The suspension is loaded onto an S-Trap filter and centrifuged to remove the flow-through. Trypsin is added to digest the proteins. The resultant peptides are eluted with three stepwise buffers (50 mM TEAB, 0.2% formic acid in water, and 50% acetonitrile + 0.2% formic acid in water) [93,94].

Several enzymes can be used for protein digestion. Among these, trypsin is the most commonly used. Trypsin cuts the polypeptide backbone at the carboxy-terminal side of lysine and arginine with high specificity, which favors the database searching step [95]. Lysine and arginine residues are frequently present in proteins; thus, digestion with trypsin results in peptides with an average length that is convenient for LC-MS/MS analysis. Since lysine and arginine are basic residues, the resultant tryptic peptides have a positive charge, usually +2 and +3. They are suitable for producing positively charged peptides with an ideal m/z in LC-MS/MS analysis [1].

2.1.4. Peptide Purification

Peptide purification is usually conducted using reversed-phase solid-phase extraction (SPE). A C18 SPE cartridge is conditioned with an organic solvent (methanol) and equilibrated with acidified water (0.1% formic acid or trifluoroacetic acid) [96]. After the peptide samples are loaded into the cartridge, contaminants are washed with washing solutions (0.1% formic acid or trifluoroacetic acid in water). The peptides retained in the cartridge are then eluted with strong solvents (e.g., 80% acetonitrile, 20% water, 0.1% formic acid) [97,98]. The eluted samples containing peptides are subjected to lyophilization or vacuum drying.

2.1.5. Sample Fractionation

Sample fractionation is an optional step that can reduce the complexity of the peptide mixture prior to LC-MS/MS analysis. Because the final LC step is usually a high-pH reversed-phase LC, an orthogonal type of LC is used for sample fractionation, such as SCX [99], anion exchange chromatography [100], size exclusion chromatography [101], or low or middle pH reversed-phase LC [102]. In high-pH reversed-phase LC fractionation, the mobile phase usually consists of acetonitrile and ammonium formate (pH 10.0) [102]. In SCX fractionation, the mobile phase is 0.1% formic acid in water and 890 mM ammonium acetate solution (pH = 2.88) [99] or 10 mM KH_2PO_4 in acetonitrile:water (pH 2.7 and 10 mM KH_2PO_4 + 0.6 M) in acetonitrile:water (pH 2.7 [74]. Sample fractionation can be performed using an offline [74] or online strategy [99]. Occasionally, three-dimensional fractionation is required to achieve an in-depth proteome profile [103].

2.2. Data Acquisition

After preparation, the samples are injected into an LC-MS/MS system for analysis. Peptides are separated using a column, typically a reversed-phase column (e.g., C18). The mobile phase usually includes a gradient of solvents (e.g., 0.1% formic acid in water and 0.1% formic acid in 80–90% acetonitrile) with an increasing percentage of the organic solvent. In reverse-phase LC, peptides are separated based on their hydrophobicity. Hydrophilic peptides are eluted earlier, followed by those with increased hydrophobicity. The

analysis time can be several minutes (for targeted proteomics) or 1–3 h (for untargeted proteomics) [104].

Peptides eluted from the column are analyzed using a mass spectrometer. Depending on the type of data acquisition, there are untargeted or targeted proteomics. Untargeted or shotgun proteomics aims to collect as much data as possible. Commonly, peptide masses are measured (precursor scan), and the top 10 or more peptides with the highest intensities are sequentially isolated and fragmented. MS/MS spectra are recorded. Untargeted proteomics is usually used to profile the proteome of samples, which can present a comprehensive view of their protein components [105]. In contrast, targeted proteomics only detects and quantifies a small number of peptides specific for selected proteins. Generally, it is carried out on a triple-quadrupole mass spectrometer using selected reaction monitoring (SRM) or multiple reaction monitoring (MRM). As shown in Figure 1, m/z values of the targeted peptides were set in the system; thus, only peptides with these predetermined m/z values were isolated (in quadrupole Q1) and fragmented (in quadrupole Q2). In quadrupole Q3, only fragment ions with a predetermined m/z value were filtered and allowed to pass through the detector. Double filtering makes SRM and MRM methods highly specific and sensitive. Targeted proteomics is appropriate for precise protein quantitation [106].

2.3. Data Analysis

Targeted proteomic data analysis is simple because all m/z values are predetermined for specific peptides. In untargeted proteomics, peptides are unknown and can be identified using different approaches. One of the most common peptide identification methods is the database search. In this method, theoretical spectra are generated from peptide sequences (database) via *in silico* digestion. Typically, search engines compare each observed spectrum to all peptides in a database that has masses within a predefined tolerance. The database search approach finds the best peptide-spectrum match (PSM) from a series of peptide candidates in the sequence database. A protein sequence database limits the space required for the software to be searched. Therefore, a database search is generally the most common approach for peptide identification when a protein sequence database is available [107]. Various software tools have been developed for database search, such as SEQUEST [108], Mascot [109], X!Tandem [110], MaxQuant [111], PEAKS DB [112], Comet [113], and MSFragger [114]. Other statistical modeling tools such as PeptideProphet [115], Percolator [116], and IDPicker [117] are usually combined with database search tools to re-score PSMs reported by these database search tools.

The second approach to identifying peptides from MS/MS spectra is a spectral library search. In this approach, a spectral library is built from MS/MS spectra of known peptides, and the observed MS/MS spectra are compared to the actual experimental spectra in the library. This method reduces the search time, but requires the establishment of a spectral library [118]. However, when using this approach, a peptide present in the samples will not be detected if its spectrum is not in the library. Spectral library searches can be performed using software tools including SpectraST [119], X!Hunter [120], M-SPLIT, QuickMod [121], Pepitome [122], and MSPepSearch [123].

Another method is *de novo* peptide identification, which uses information in MS/MS spectra to determine peptide sequences without using sequence databases. It considers all possible amino acid combinations, and thus, the search space is remarkably larger than that of the database search. As a result, *de novo* sequencing is slower than the other approaches. Using this method, multiple sequences are generally derived at equal probabilities for each spectrum. *De novo* sequencing is generally not used as a primary tool to identify peptides from MS/MS spectra. It is useful when the species is unknown, or a sequence database is not available. *De novo* sequencing can also be applied after database search to determine unassigned peptide sequences [124]. Some software tools for this approach are LuteFisk [125], Sherenga [126], PEAKS [127], PepNovo [128], NovoHMM [129], and DeepNovo [130].

3. Applications of Forensic Proteomics for Human Samples

3.1. Hair Proteome

Hair is often available at crime scenes. It is formed by keratinization of epidermal keratinocytes. Hair is physically flexible and robust due to its components, which are primarily coiled-coil proteins with intermolecular disulfide [131]. It generally persists under different environmental conditions. However, hair may not be a favorable sample for DNA analysis because DNA can be extensively degraded due to the keratinization process [132]. A proteomic approach can be performed using hair for individual identification. Notably, mitochondrial genome and proteome profiles have been obtained from hair samples using a combined workflow with high compatibility [133]. A recent study proposed a gel-based proteomic approach to analyze hair samples with high sensitivity, and built a library containing all identified peptides derived from hair [134].

In forensic science, hair can be used in different applications. In a previous study on human hair, Laatsch et al. used shotgun proteomics (i.e., bottom-up proteomics using LC-MS/MS) to identify ethnic-based differential proteomic signatures among different ethnic groups, including Caucasians, African-Americans, Kenyans, and Koreans [36]. It was found that differences among ethnic groups mainly relied on the levels of keratin-associated proteins in hair (Figure 2a). A recent study demonstrated that keratin peptides of human hair could be used to distinguish gender (using type II-keratin K81, K83, and K86 peptides) and ethnicity (using type I and type II keratin K33b, K81, K83, and K86 peptides) [26]. Another study reported the use of shotgun proteomics to characterize hair shaft proteins in 66 European-American subjects to obtain individualizing and biogeographic information such as ethnicity [18]. Genetically variant peptides (GVPs) containing single amino acid polymorphisms (SAPs) were found in the proteomic datasets and then used to impute the status of non-synonymous single nucleotide polymorphisms (nsSNPs) alleles in subject genomes. It should be noted that mitochondrial DNA and nsSNPs have been used to identify ethnicity, race, and gender [135]. GVPs containing SAPs have been determined by shotgun proteomics for the same purpose because they result from these DNA nsSNPs [136]. In the study by Parker et al., the probability of detecting nsSNP alleles or allele combinations in each gene ($\text{Pr}(\text{nsSNP gene profile} | \text{population})$) was calculated and multiplied together to estimate the probability of an overall individual nsSNP profile in the population ($\text{Pr}(\text{profile} | \text{population})$) [18]. Thus, European-American and African-American subjects could be differentiated by imputed nsSNP profiles (Figure 2b). In addition, likelihood ratios (LRs) for European relative to African populations were calculated for each individual: $\text{LR}(\text{EUR}/\text{AFR}) = \text{Pr}(\text{profile} | \text{EUR population}) / \text{Pr}(\text{profile} | \text{AFR population})$. As shown in Figure 2c, the European-American hair shaft protein samples had LRs of 6.50×10^{-1} to 5.85×10^3 , which were higher than those from African-American and Kenyan samples (1.15×10^{-3} to 1.07×10^1 and 9.9×10^{-3} to 1.21×10^1 , respectively). The findings in this study suggest great potential of imputed nsSNP allele profiles obtained from hair shaft proteins in determining the biogeographic ancestral background of individuals with quantifiable statistical information. Recently, this group developed and optimized a proteomic workflow to allow individual identification from a single human scalp hair with a length of 2 cm [37]. The optimal conditions were as follows: (1) reduction with 100 mM dithiothreitol at room temperature for 6 h, (2) alkylation with 200 mM iodoacetamide for 45 min, and (3) tryptic digestion (enzyme:protein ratio of 1:50, addition twice at 0 h and 3 h) at room temperature. The authors demonstrated that this optimized strategy increased the total identified genetically variant peptides from 45 to 127, and the sensitivity of the analysis 3-fold from 11% to 34%. The optimal condition contributed to increase LRs (AFR/EUR) for African samples about $10^{3.9}$ times. Another study showed the feasibility of extracting and determining proteins from a single hair with a length of approximately 1 mm [137]. Using the proteomic approach, 63 proteins were identified, of which 60% were keratin and keratin-associated proteins. However, no data have been presented regarding genetically variant peptides. A later study by Mason et al. developed a new proteomic sample preparation procedure for a single hair (length, 25 mm),

which combined heat, ultrasonication, and surfactants [38]. A total of 6519 unique peptides and 57 GVPs were identified, demonstrating the forensic application of this approach to distinguish individuals.

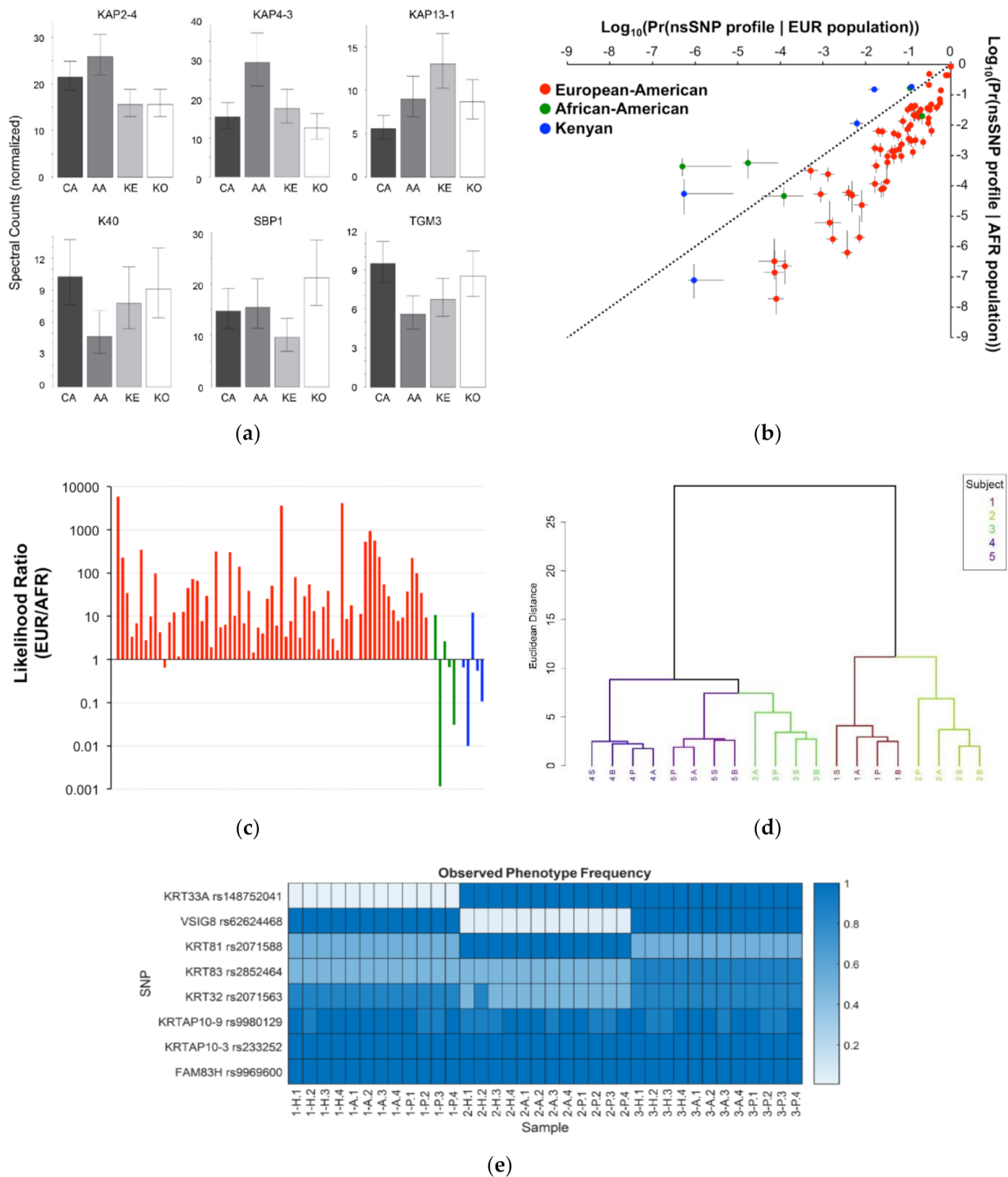


Figure 2. Applications of human hair proteomic analysis in forensic sciences. **(a)** Weighted spectral counts for six differentially expressed proteins that were in hair samples of subjects of different ethnic origin. The ethnicities are Caucasian (CA), African-American (AA), Kenyan (KE), and Korean (KO). Figure reprinted from [36] under the terms of the Creative Commons Attribution

License (CC BY 4.0) <https://creativecommons.org/licenses/by/4.0/> (accessed on 3 March 2021). (b) Identification of individuals and differentiated biogeographic backgrounds using non-synonymous single nucleotide polymorphism (nsSNP) imputed from single amino acid polymorphisms (SAPs) in human hair proteomes. The probability of overall imputed nsSNP profiles occurring in the European or African population ($\Pr(\text{profile} | \text{EUR population})$ or $\Pr(\text{profile} | \text{AFR population})$) was calculated using imputed nsSNP alleles from individuals and multiplied together. Confidence intervals (90% CI) are estimated using parametric bootstrapping. European and African populations were differentiated by their probability of overall imputed nsSNP profiles using parametric bootstrapping. Figure reprinted from [18] under the terms of the Creative Commons Attribution License (CC BY 4.0) <https://creativecommons.org/licenses/by/4.0/> (accessed on 3 March 2021). (c) The likelihood of hair samples coming from a European relative to African genetic background. The colors are for European-American subjects (red), African-American subjects (green), and Kenyan subjects (blue). Figure reprinted from [18] under the terms of the Creative Commons Attribution License (CC BY 4.0) <https://creativecommons.org/licenses/by/4.0/> (accessed on 3 March 2021). (d) Hierarchical clustering dendrogram of identified genetically variant peptides (GVPs), which indicated that GVPs of hair shaft were more related to individual differences than the body site origin. Subjects are shown in different colors. Sample labels indicate hair body site origin: S = Scalp, B = Beard, P = Pubic, A = Axillary. Figure reprinted from [39], Copyright (2019), with permission from Elsevier. (e) GVP profiles of 36 samples at 8 SNP loci using observed phenotype. Sample code: the first number was for 3 subjects, the second letter was for hair body site origin (H: head, A: arm, and P: pubic), and the third number was for 4 samples originated from a hair. Figure reprinted from [27] under the terms of the Creative Commons Attribution License (CC BY 4.0) <https://creativecommons.org/licenses/by/4.0/> (accessed on 3 March 2021).

A recent study compared the proteome profiles of human hair from different body sites and individuals [39]. The study revealed that hair GVPs could be used to distinguish individuals without concern for hair body site origin (Figure 2d). Similarly, Chu et al. determined eight SNPs corresponding to GVPs that could differentiate individuals using a single hair regardless of its body location origin [27]. The GVP profiles in Figure 2e demonstrate high inter-individual variation as well as high intra-individual consistency. A recent study also revealed that hair GVP profiles were robust regardless of hair pigmentation status [138]. Parker et al. also confirmed that GVP was not affected by the anatomical origin of the hair shaft and hair pigmentation [139]. Chu et al. demonstrated that hair damaged under harsh conditions (e.g., fires) could still be used to determine GVP profiles [140]. Another study by Plott et al. showed the robustness of hair GVP profiles over periods as long as 65 years [141].

3.2. Bone Proteome

Bone proteins are present in the extracellular matrix and mostly consist of collagenous proteins (~90%) [142]. Bone collagens are extremely stable due to their cross-linked structure and the protection of the bone matrix [143]. Thus, Buckley et al. determined a peptide with 33 amino acids derived from collagen, which could be used to distinguish between sheep and goat bone because this peptide differs between the two species at two positions [144]. A study by Wadsworth et al. found that while many low-abundance proteins in bone degraded with time, serum albumin, alpha-2-HS-glycoprotein (AHSG) could be easily recovered in ancient bone [145]. Other proteins such as lumican, chondroadherin, and biglycan also survive well because of their interactions with bone collagen. They can be useful for identifying species and studying phylogenetic inferences in archeological and paleontological bones.

Several studies have investigated bone proteomes to estimate biological age (age-at-death) and PMI of skeletal tissue in simulated forensic contexts. Procopio et al. studied the relationship between biological age (age-at-death) and the abundance of some proteins in proteomic analysis of pig bones [40]. They found that AHSG decreased with age. In addition, two serum proteins (alpha-1 antitrypsin and chromogranin-A) positively correlated with biological age (Figure 3a). This group later reported a similar relationship between AHSG abundance and the biological age of human 4000–5500-year-old ancient mandibular bone [41]. Sawafuji et al. revealed a strong negative correlation between the abundance of AHSG in human bone samples and the biological age of the specimens [42]. The authors used the exponentially modified protein abundance index (emPAI) score [146] and established a relationship between the normalized emPAI score of AHSG and age.

A strong negative correlation was demonstrated by Pearson's r of -0.9826 , p -value of 1.295×10^{-5} , and Bonferroni-corrected p -value of 5.95×10^{-4} (Figure 3b). In addition, the study showed that the normalized emPAI scores of albumin, kininogen-1 (KNG1), insulin-like growth factor-binding protein 5 (IGFBP5), and pigment epithelium-derived factor (SERPINF1) were significantly correlated with age.

Procopio et al. later performed a proteomic study on porcine bones to investigate both PMI and biological age [29]. They revealed the stability of AHSG after death in forensic contexts without significant inter-individual variability, as well as the correlation between its relative abundance and the biological age of an individual. AHSG has a great affinity to the mineral matrix of the bone, and thus is protected and can be recovered after hundreds of years. Based on these findings, the authors suggested that AHSG could be a potential biomarker for the estimation of age at death. The study also found that the abundance of some plasma and muscle proteins (hemoglobin A and B, transferrin, lactoferrin, haptoglobin, creatine kinase M-type, myosin type 2 and 6) reduced quickly within 4 months of PMI. Notably, asparagine deamidation of biglycan (a protein with an important role in modulating bone growth and mineralization) increased consistently with PMI (Figure 3c) and could be useful for identifying specific PMIs (from one to six months).

Prieto-Bonete et al. performed proteomic analysis on 40 femur bones from 40 different cadavers and selected 32 proteins that allowed discrimination between PMI of 5–12 years and PMI of 13–20 years [30]. They included 25 non-collagenous proteins (ADAMTS17, ANXA2R, AREG, BMP5, CD163L1, CDH11, CTC1, CTNNB, DLX5, ENPP1, FBN1, FGFR1, INSIG2, JAG2, LAMA2, LMNA, MAF, MED12, MUC15, MYSM1, PAPP, PHOSPHO1, SLC26A1, SOX6, SUCO) and seven collagen proteins (CILP, COL10A1, COL11A2, COL5A2, COL9A2, COMP, and PCOLCE). Mizukami et al. recently examined the relationship between PMI and the proteome of mouse bones under aquatic decomposition in four different types of aquatic environments, including tap water, saltwater, pond water, and chlorinated water [147]. They found that the effects of PMI on protein abundance were superior to those of the aquatic environment. Notably, muscle protein fructose-bisphosphate aldolase A (ALDOA) showed decreases in abundance with increasing PMI, whereas protein peptidyl-prolyl cis-trans isomerase (PPIA) and coagulation factor VII (F7A) could be potential biomarkers for distinguishing samples between terrestrial and aquatic environments. AHSG was used to potentially identify samples in pond water because it suffered significant deamidation in this environment compared to other aquatic environments.

A recent study evaluated the proteome of whole skeletal elements using rats and determined protein biomarkers to estimate biological age [148]. They found five newly identified proteins that could be used for age estimation, including vimentin, osteopontin, matrilin-1, apolipoprotein A-I, and prothrombin. While the abundance of prothrombin increased with age, those of vimentin, osteopontin, matrilin-1, and apolipoprotein A-I showed an inverse trend (Figure 3d). Thus, these proteins, along with previously reported proteins (AHSG or fetuin-A, alpha-1 antitrypsin, chromogranin-A, albumin, KNG1, IGFBP5, SERPINF1, and biglycan) have potential for development of an age estimation tool for skeletal samples in forensic contexts.

Similar to human hair samples, human bone samples could be used to identify SAPs for human identification, although there have been few reports on this application. Mason et al. recently performed proteomic analysis on human rib cortical bone samples and identified 15 proteins containing GVPs [43]. GVPs were correlated to unique DNA loci, and a total of 134 inferences were made. LR values were in the range of 1.4–825 with a median value of 16. GVPs obtained from the bone proteome can be effective in inferring SNP alleles, particularly when DNA is damaged. They can be used to match samples to ethnic groups and can be applied to human identification.

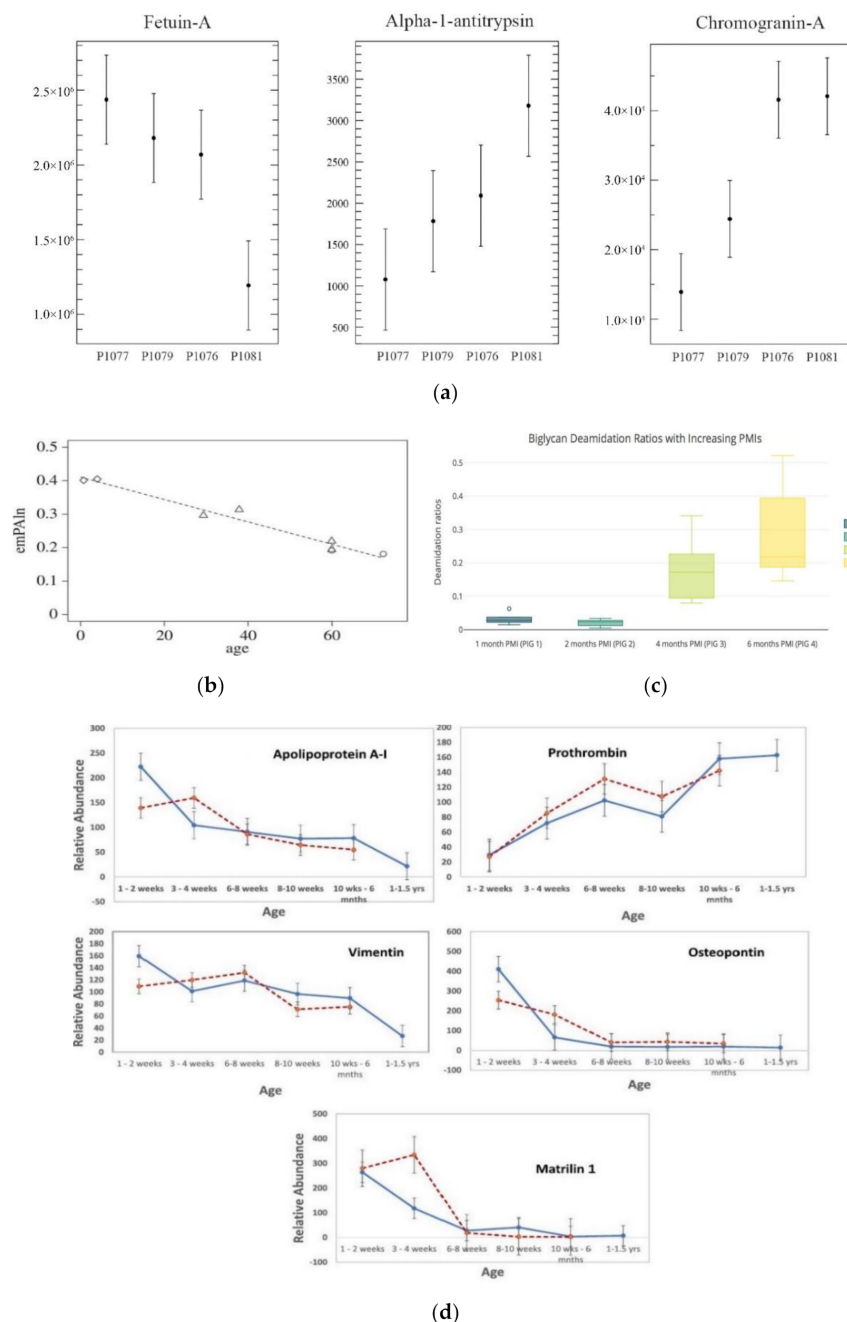


Figure 3. Applications of bone proteomic analysis for estimation of biological age and post-mortem interval (PMI). **(a)** Boxplots of relative abundances of fetuin-A (AHSG), alpha-1-antitrypsin, and chromogranin-A in proteomic analysis of porcine tibia midshaft samples with varying biological age of pigs (P1077—12 days old, P1079—28-days old, P1076—43-days old, and P1081—10-months old). Figure was adapted with permission from [40]. Copyright (2017) American Chemical Society. **(b)** Relationship between age and the normalized emPAI value of alpha-2-HS-glycoprotein (AHSG), Pearson's $r = -0.9826$, $p\text{-value} = 1.295 \times 10^{-5}$. Symbols are □ (infant), ○ (adult female), and Δ (adult male). Figure was reprinted from [42], under the terms of the Creative Commons Attribution License (CC BY 4.0) <https://creativecommons.org/licenses/by/4.0/> (accessed on 3 March 2021). **(c)** Box plot representing deamidation ratios calculated for the four tibias collected after one, two, four, and six months PMI. Each box represents five replicates that have been analyzed per individual. Figure was reprinted from [29], Copyright (2018), with permission from Elsevier. **(d)** Average abundance (calculated using label-free quantification) of apolipoprotein A-1, prothrombin, vimentin, osteopontin, and matrilin-1 for male (blue solid lines) and female (red dashed lines) rats (1 week—1.5-years old). Figure was adapted with permission from [148]. Copyright (2020) American Chemical Society.

3.3. Identification of Body Fluids and Tissues

Conventional DNA analysis can determine the donor's identity from human body fluids or tissues collected at crime scenes. However, a genetic profile alone cannot distinguish the body fluid or tissue type of samples (e.g., whether the fluids are blood, saliva, vaginal fluid, semen, or menstrual blood). Identifying specific body fluids or tissue sources can provide critical information on the context in which the samples may have been deposited [149]. Forensic serology is the study of classifying human body fluids, which relies on chemical reaction assays, enzyme activity assays, immunoassays, and microscopy-based assays [150]. For example, specific antibodies can determine the hemoglobin of blood, alpha amylase of saliva, semenogelin, and other prostate-specific antigens of semen. However, these tests are often presumptive, and they are usually unable to make a definite statement about the body fluid or tissue origin of a sample [12]. To achieve the final conclusion of the human body fluid type, a sample may be subjected to multiple tests, which is challenging for a limited sample amount [150]. Thus, different novel technologies have been developed, including the use of mRNA and miRNA, epigenetics, spectroscopic techniques (Raman spectroscopy, Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, and fluorescence spectroscopy), biosensors, and proteomics [151]. Proteomics has emerged as a powerful and feasible strategy, allowing detection of various proteins in a single experiment and requiring only a small amount of sample. Notably, sample preparation procedures for targeted proteomics and conventional DNA analysis are highly compatible [152].

To determine the identity of body fluids, untargeted proteomics is performed, as depicted in Figure 4a. This workflow includes the extraction of proteins from materials, protein digestion, and LC-MS/MS analysis of peptides. Based on the proteomic results, one can use a decision tree (Figure 4b) developed by Van Steendam et al. in 2003 to identify which body fluids are available [14]. This decision tree is based on a set of marker proteins for different biological fluids. Hemoglobin (alpha and beta subunit) is present in blood in large amounts and with high specificity, and is thus used as a biomarker of blood [153]. In addition, proteomic analysis can determine species-specific peptides, which allow distinguishing between different species of origin. A previous study tested three species, including *Homo sapiens*, *Canis familiaris*, and *Bos taurus*, and unambiguously identified species-specific peptides [154]. Marker proteins of semen are semenogelin 1 and 2, prostate-specific antigen, and prostatic acid phosphatase, while cornulin, involucrin, and cornifin are vaginal biomarkers. Alpha-amylase 1 is present in saliva, and alpha-amylase 2 is present in semen and vaginal secretion; thus, alpha-amylase 1 is chosen as a salivary marker protein because of its high abundance and specificity. Biomarkers of menstrual blood are hemoglobin from blood and cornulin from vaginal secretions [14]. Plunc-protein is specific for nasal secretions and is therefore selected as a biomarker for this matrix [155]. Uromodulin is the most abundant protein in normal human urine, whereas alpha-1-microglobulin/bikunin precursor (AMBP) can also be detected in urine. Since these proteins are found in blood, their presence in the absence of hemoglobin can be used to identify urine [156,157]. However, it should be noted that uromodulin and AMBP are sometimes present in urine at low concentrations and cannot be detected by proteomic analysis. Immunoglobulins can be detected in feces and can be used as a marker protein of this matrix together with the absence of hemoglobin [14]. Using this decision tree, Van Steendam et al. were able to correctly identify menstrual blood, peripheral blood, semen, vaginal fluid, saliva, nasal secretion, urine, and feces. They could also detect several mixtures of these fluids using simulated samples (such as semen + vaginal fluid, semen + human blood, semen + saliva, human blood + saliva, and bovine blood + saliva) and real forensic samples [14]. However, this technique has some disadvantages, including low specificity and inconsistent detection of several candidate biomarkers for a given target body fluid and potential detection of high-specificity protein biomarkers in non-target body fluids. Thus, other studies have been carried out to solve these limitations, such as

the use of various protein biomarkers [12], ratios of different proteins, or machine-learning approaches [4].

In 2014, Legg et al. performed untargeted proteomic analysis to identify 29 candidate protein biomarkers of human peripheral blood, menstrual fluid, semen, vaginal fluid, saliva, and urine [158]. This group later conducted a targeted proteomics analysis to validate these proteins in a larger cohort of volunteers [12]. Table 2 presents biomarker candidates with peptide targets for each body fluid. This group evaluated the specificity of each biomarker candidate for each body fluid using a multiplexed Q-TOF assay on body fluids of 50 males and 50 females. Table 3 shows an estimation of the frequency at which target biomarkers may be detected in target and non-target body fluids. The multiplex assay also accurately identified two components in different 2-component mixtures of human body fluids, except in the case of saliva and peripheral blood [12]. A recent study applied machine-learning techniques to protein abundance data and distinguished different tissues and organs with high accuracy [159].

Results were obtained from the targeted ion Q-TOF multiplex assays, with $n = 50$ saliva, seminal fluid, vaginal/menstrual fluid, and peripheral blood, and $n = 25$ for female urine and male urine. Each body fluid (listed in the top row) was tested for various targeted biomarkers (listed on the left side). Values are the percentage of samples in which the target protein marker was confidently identified (at least one target peptide per protein, using a validated database search). Table adapted with permission from [12]. Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Table 2. Biomarker candidates including peptide targets.

Fluid	Protein Biomarker	Accession Number	Peptide Sequence
Peripheral Blood	Hemoglobin subunit beta *	P68871	LLVVYPWTQR VVAGVANALAHKYH GTFATLSELHCDK
	Complement C3 *	P01024	TMQALPYSTVGNSSNNYLHLSVLR VYAYNLEESCTR VFLDCCNYITELR
	Hemopexin *	P02790	NFSPVDAAFR YYCFQGNQFLR
Saliva	Cystatin SA *	P09228	IIEGGIYDADLNDER SQPNLDTCAFHEQPELQKK QLCSFQIYEVWEDR
	Cystatin D *	P28325	SQPNLDCNCFNDQPK TLAGGIHATDLNDK
	Submaxillary gland androgen regulated protein *	P02814	GPYPGGLAPPQPFPGFVPPPPPPYGPGR IPPPPPAPYGPFI PPPPPQ
	Statherin *	P02808	FGYGYGPYQPVPEQPLYQPQYQYQYTF
	Histatin-1	P15515	EFPPYGDYGSNYLYDN
Seminal fluid	Semenogelin 1 *	P04279	KQGSQSSYVLQTEELVANK DIFTTQDELLVYNK
	Semenogelin 2 *	Q02383	DVSQSSISFQIEK DIFTTQDELLVYN
	Prostate-specific antigen	P07288	VMDLPTQEPALGTTTCYASGWGSEPEEFLTPK AVCGGVLVHPQWVLTAAHCIR LSEPAELTDAVK
	Prostatic acid phosphatase	P15309	ELSELSSLISLYGIHK FQELSETLKSEEFQK SPIDTFPTDIK
	Glycodelin	P09466	VHITSLPTPEDNLEIVLHR VLVEDDEIMQGFIR
	Epididymal secretory protein E1	P61916	AVVHGILMGVVPVPIPEPDGCK EVNVSPCPTQPCQLSK
Urine	Osteopontin *	P10451	GDSVVYGLR QLYNKYDAVATWLNPDPSQK AIPVAQDLNAPSDWDSR
	Uromodulin *	P07911	VLNLGPITR STEYGEYACDITDLR DGPCGTVLTR

Table 2. Cont.

Fluid	Protein Biomarker	Accession Number	Peptide Sequence
Vaginal/ Menstrual	Mucin 5B/Cervical	Q9HC84	GYQVCPVLADIECR AQAQPGVPLGELGQVVECSLDFGLVCR AAGGAVCEQPLGLECR ISPQIQLSGQTEQTQK
	Cornulin *	Q9UBG3	TLSESAEGACCSQESGLHSGASQELGEGQR APGWDPLCWDECR
	IgGfC-binding protein *	Q9Y6R7	SLAAYTAACQAAGVAVKPWR AGCVAESTAVCR DGVTPGPGFTLSGCCQCSR GLDLHGLLAFLQLQQCAQDR GCVQDEFCTR
	Ly6/PLAUR-containing protein 3	O95274	GSRPQGPFLLADKWPALPR SYPLTSLYLR
	Matrix metalloproteinase-9	P14780	TFVPGCQPGFTLGNK ALDGINSGITHAGR
	Neutrophil gelatinase *	P80188	LGQGVNHAADQAGKEVEK
	Suprabasin	Q6UWP8	

* Confirmatory biomarkers. Table adapted with permission from [12]. Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Table 3. Estimation of frequency at which target biomarkers may be detected in target and non-target body fluids.

Protein Biomarker		Fluid 1/2pt Line					
		Semen	Saliva	Male Urine	Female Urine	Vaginal Fluid	Menstrual Fluid
Semen	Semenogelin 1	100%		24%			
	Semenogelin 2	100%		20%			
	Epididymal secretory protein E1	100%		40%	30%	2%	
	Prostate-specific antigen	100%		60%			
	Prostatic acid phosphatase	100%		80%			
Urine	Glycodelin	82%					16%
	Uromodulin			100%	100%		
	Osteopontin			100%	100%		
Saliva	Submaxillary gland androgen regulated protein		100%				
	Cystatin D		76%				
	Cystatin SA		94%				
	Statherin		90%				
	Histatin-1		30%				
	Mucin 5b		96%	4%	4%	38%	20%
Vaginal/ Menstrual	Cornulin				4%	100%	20%
	Neutrophil gelatinase					100%	6%
	Ly6/PLAUR-containing protein 3				4%	100%	4%
	IgGfC-binding protein					68%	
	Matrix metalloproteinase-9					20%	
	Suprabasin					22%	
Peripheral Blood	Hemoglobin subunit beta		4%		12%	6%	100%
	Complement C3						54%
	Hemopexin				6%	4%	76%

In addition to proteomics, peptidomics can also be applied to analyze peptides that are naturally present in body fluids without enzymatic digestion. These peptides are usually derived from endogenous proteolytic enzymes, and thus their masses and charges may vary over a wide range, which causes difficulties in conventional proteomic database searching. Several programs have been developed for peptide mapping, such as PatternLab [160], iPiG [161], SWPepNovo [162], and Peptigram [163]. Based on the above-mentioned successes of forensic proteomics in the identification of body fluids, peptidomics may also be exploited to determine potential peptide biomarkers in these body fluids and can be widely applied in forensic sciences [164]. A recent study reported the reliable recovery and detection of a small amount (0.5–5.0 µL) of seminal fluid on cotton swabs [165]. Seminal fluid peptide biomarkers were also determined, which correspond to well-known seminal protein biomarkers, such as semenogelin I and II, prostate-specific antigen, and prostatic acid phosphatase.

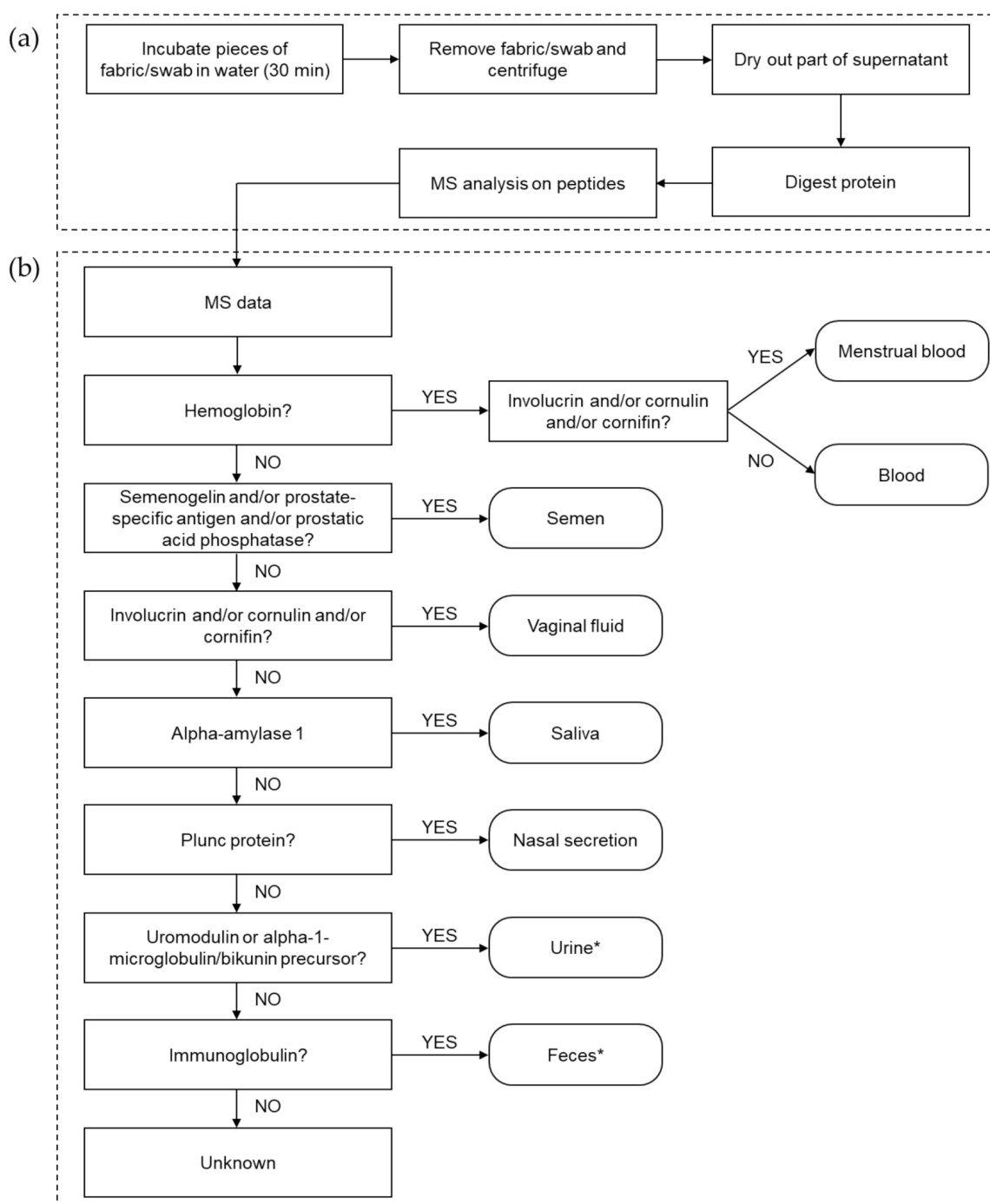


Figure 4. Identification of biological matrices in forensic proteomics. **(a)** Workflow for mass spectrometry-based identification of biological matrices. **(b)** Decision tree with biomarkers per biological matrix. * The absence of the biomarker (uromodulin/alpha-1-microglobulin/bikunin precursor protein or immunoglobulins) does not necessarily exclude the presence of the matrix (urine or feces, respectively). Figures redrawn from [14], under the terms of the Creative Commons Attribution 2.0 International License (CC BY 2.0) <https://creativecommons.org/licenses/by/2.0> (accessed on 3 March 2021).

Kennedy et al. devised a MALDI MS-based proteomic strategy that could reliably detect blood, discriminate human and animal blood, determine animal blood species, and identify semen (Figure 5) [44]. This approach can be used for the detection and provenance of blood with easy data acquisition, user-friendly data interpretation, and short analysis time. However, it is unable to detect various body fluids, as previously described.

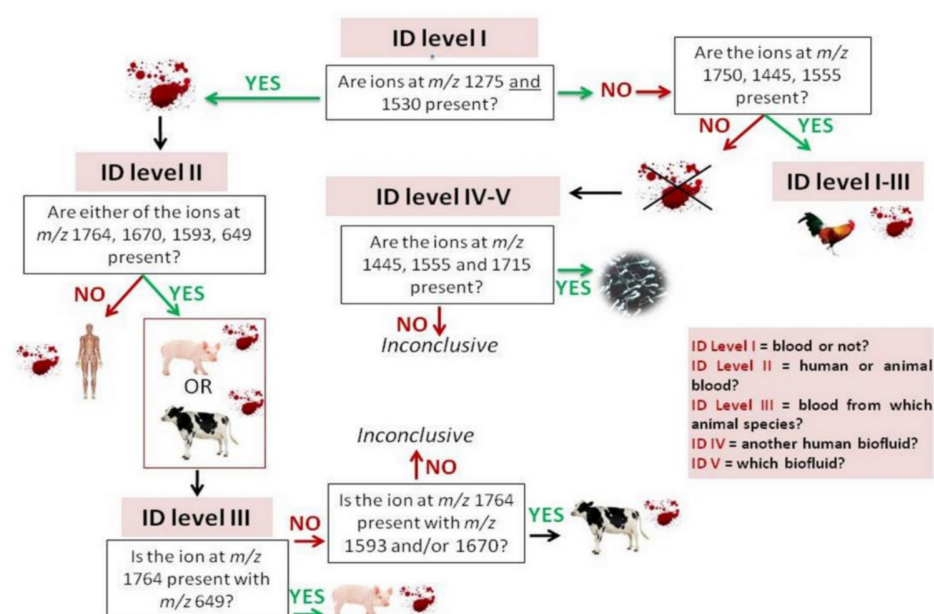


Figure 5. Interpretative strategy to identify blood samples (from human and animal species) and semen using matrix assisted laser desorption ionization mass spectrometry (MALDI MS). Identification (ID) levels I–III were for animal provenance (down to species) and ID levels IV–V were for determination of semen. Reprinted from [44], under the terms of the Creative Commons Attribution License (CC BY 4.0) <https://creativecommons.org/licenses/by/4.0/> (accessed on 3 March 2021).

Forensic proteomics is also applicable for organ identification. For instance, Dammeier et al. performed proteomic analysis of tissue residue from bullets in an attempt to determine which organs had been penetrated by a certain bullet [45]. In this study, the bullets were fired into or pressed through different bovine organs (kidney, lung, liver, heart, and skeletal muscle). Subsequently, the tissue residues were collected and subjected to proteomic analysis. The lists of identified proteins were fed to a machine learning algorithm to predict the relevant organs. The method, however, succeeded only in the test cases. In actual homicide cases, some bullets passed through multiple organs; thus, the method sometimes failed to predict the organs. Nevertheless, the study found highly discriminating proteins in different organs, as presented in Table 4. The proteomic analysis of the tissue residue proposed in this study can be further investigated for future applications.

Table 4. Highly discriminating protein identifications per organ (top 8).

Protein Name	Accession (Bovine)	Accession (Human)	Pearson Correlation Coefficient
Heart			
Myosin binding protein C, cardiac-type	Q0VD56_BOVIN	MYPC3_HUMAN	0.92
Glycogen phosphorylase, brain form	PYGB_BOVIN	PYGB_HUMAN	0.83
Troponin I, cardiac muscle	TNNI3_BOVIN	TNNI3_HUMAN	0.79
Myosin light chain 3	MYL3_BOVIN	MYL3_HUMAN	0.76
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	ODPB_BOVIN	ODPB_HUMAN	0.75
Calpastatin	Q9XSX1_BOVIN	ICAL_HUMAN	0.74
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	NDUA4_BOVIN	NDUA4_HUMAN	0.74
Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	COX41_BOVIN	COX41_HUMAN	0.70

Table 4. Cont.

Protein Name	Accession (Bovine)	Accession (Human)	Pearson Correlation Coefficient
Kidney			
Calbindin	CALB1_BOVIN	CALB1_HUMAN	1
Na(+)/H(+) exchange regulatory cofactor NHE-RF3	NHRF3_BOVIN	NHRF3_HUMAN	1
Low-density lipoprotein receptor-related protein 2	F1N6H1_BOVIN	LRP2_HUMAN	1
Villin 1	Q5E9Z3_BOVIN	VILI_HUMAN	0.96
Retinyl ester hydrolase type 1	Q5MYB8_BOVIN	Q8TDZ9_HUMAN	0.92
Membrane metalloendopeptidase variant 2	E1BPL8_BOVIN	NEP_HUMAN	0.92
Phosphotriesterase related protein	PTER_BOVIN	PTER_HUMAN	0.83
Plastin-1	PLSI_BOVIN	PLSI_HUMAN	0.79
Liver			
Hydroxymethylglutaryl-CoA synthase, mitochondrial	HMCS2_BOVIN	HMCS2_HUMAN	0.96
Carbamoyl-phosphate synthase [ammonia], mitochondrial	F1ML89_BOVIN	CPSM_HUMAN	0.92
Phenylalanine-4-hydroxylase	PH4H_BOVIN	PH4H_HUMAN	0.92
3-oxo-5-beta-steroid 4-dehydrogenase	E1BBT0_BOVIN	AK1D1_HUMAN	0.92
Catechol O-methyltransferase	COMT_BOVIN	COMT_HUMAN	0.92
Acetyl-CoA acetyltransferase	Q17QI3_BOVIN	THIC_HUMAN	0.92
Cytochrome P450 2E1	CP2E1_BOVIN	CP2E1_HUMAN	0.92
Dimethylaniline monooxygenase	G5E5R0_BOVIN	FMO1_HUMAN	0.92
Lung			
Plastin-2	F1MYX5_BOVIN	PLSL_HUMAN	0.93
Cathelicidin-4	CTHL4_BOVIN	CAMP_HUMAN	0.90
Tubulin beta chain	TBB5_BOVIN	TBB5_HUMAN	0.90
Cysteine and glycine-rich protein 1	CSRP1_BOVIN	CSRP1_HUMAN	0.87
Prostaglandin F synthase 2	PGFS2_BOVIN	AK1C1_HUMAN	0.87
Myosin light polypeptide 6	MYL6_BOVIN	MYL6_HUMAN	0.84
Calpain-2 catalytic subunit	CAN2_BOVIN	CAN2_HUMAN	0.83
Alpha-actinin-1	ACTN1_BOVIN	ACTN1_HUMAN	0.83
Muscle			
Bridging integrator 1	Q2KJ23_BOVIN	Q9BTH3_HUMAN	1.00
Myosin-binding protein C, slow-type	A6QP89_BOVIN	MYPC1_HUMAN	1.00
Fructose-1,6-bisphosphatase isozyme 2	F16P2_BOVIN	F16P2_HUMAN	1.00
Myosin-binding protein C, fast-type	E1BNV1_BOVIN	MYPC2_HUMAN	1.00
Troponin C, skeletal muscle (fast type)	Q148C2_BOVIN	TNNC2_HUMAN	1.00
Myosin regulatory light chain 2, skeletal muscle isoform	MLRS_BOVIN	MLRS_HUMAN	0.96
Nebulin	F1MQI3_BOVIN	NEBU_HUMAN	0.92
PDZ and LIM domain protein 3	PDLI3_BOVIN	PDLI3_HUMAN	0.92

Protein identifications were ranked by the absolute Pearson correlation coefficient between the presence of protein identification and organ affiliation over all samples. Accessions of homologous human proteins were determined by executing the BLAST algorithm in a protein-to-protein setting. Table adapted with permission from [45]. Copyright (2016), American Chemical Society.

By applying proteomic techniques, protein biomarkers of body fluids and tissues will continue to be determined, which may have potential applications in forensics. A recent study performed label-free LC-MS/MS for proteomic profiling of 117 samples from 46 normal tissues and organs at autopsy [46]. The authors combined previous proteomic data of seven biological fluids (urine, seminal plasma, cervical vaginal fluid, sweat, cerebrospinal fluid, synovial fluid, and nipple aspirate fluid) and generated MS/MS spectra corresponding to 13,028 unique human protein-coding genes. This dataset is an important complementary resource for determining protein biomarkers for each body fluid and tissue.

3.4. Other Applications of Forensic Proteomics for Human Samples

3.4.1. Targeted Analysis of Proteins and Peptides in Urine and Blood for Sport Doping

Proteomics can be employed to detect the presence of illegal peptides and small protein hormones in urine and blood samples of athletes. They include peptide hormones, growth factors, related substances, and mimetics, as presented in the 2021-updated list of prohibited substances issued by the World Anti-Doping Agency (Table 5).

Table 5. List of prohibited peptides and proteins issued by World Anti-Doping Agency (2021).

Group	Examples
Erythropoietins (EPO) and agents affecting erythropoiesis	EPO-receptor agonists: Darbepoietins (dEPO) EPO EPO-based constructs (EPO-Fc, methoxy polyethylene glycol-epoetin beta (CERA))EPO-mimetic agents and their constructs (CNTO-530, peginesatide)
	Hypoxia-inducible factor (HIF) activating agents: Cobalt Daprodustat (GSK1278863) IOX2 Molidustat (BAY 85-3934) Roxadustat (FG-4592) Vadadustat (AKB-6548) Xenon
	GATA inhibitors: K-11706
	Transforming growth factor -beta (TGF- β) signaling inhibitors: Luspatercept, sotatercept
	Innate repair receptor agonists: Asialo EPOCarbamylated EPO (CEPO)
Peptide hormones and their releasing factors	Chorionic gonadotrophin (CG) and luteinizing hormone (LH) and their releasing factors in males: Buserelin Deslorelin Gonadorelin Goserelin Leuprorelin Nafarelin Triptorelin
	Corticotrophins and their releasing factors: Corticorelin
	Growth hormone (GH), its fragments and releasing factors: GH fragments: AOD-9604 and hGH 176-191 GH-releasing hormone (GHRH) and its analogues: CJC-1293, CJC-1295, sermorelin and tesamorelin GH secretagogues (GHS): lenomorelin (ghrelin), anamorelin, ipamorelin, macimorelin and tabimorelinGH-releasing peptides (GHRPs): alexamorelin, GHRP-1, GHRP-2 (pralmorelin), GHRP-3, GHRP-4, GHRP-5, GHRP-6, and examorelin (hexarelin)
Growth factors and growth factor modulators	Fibroblast growth factors (FGFs) Hepatocyte growth factor (HGF) Insulin-like growth factor 1 (IGF-1) and its analogues Mechano growth factors (MGFs) Platelet-derived growth factor (PDGF) Thymosin- β 4 and its derivatives e.g., TB-500 Vascular endothelial growth factor (VEGF)

A number of banned performance-enhancing peptides or proteins have been identified and quantified using LC-MS/MS assays (targeted proteomics), such as recombinant human EPO variants [166], leuprolide [167], insulin therapeutic analogs [28,47], and insulin-like growth factor-binding proteins (IGFBPs) [48]. Analysis of peptides and proteins using LC-MS/MS assays is highly selective compared to traditional immunological techniques [168]. Targeted proteomics can identify peptides and proteins with similar structures that may not be distinguished using antibody-based strategies. It can also simultaneously identify and quantify many peptides and proteins, such as IGFBP1, 2, 3, and 5 (Figure 6a) [48].

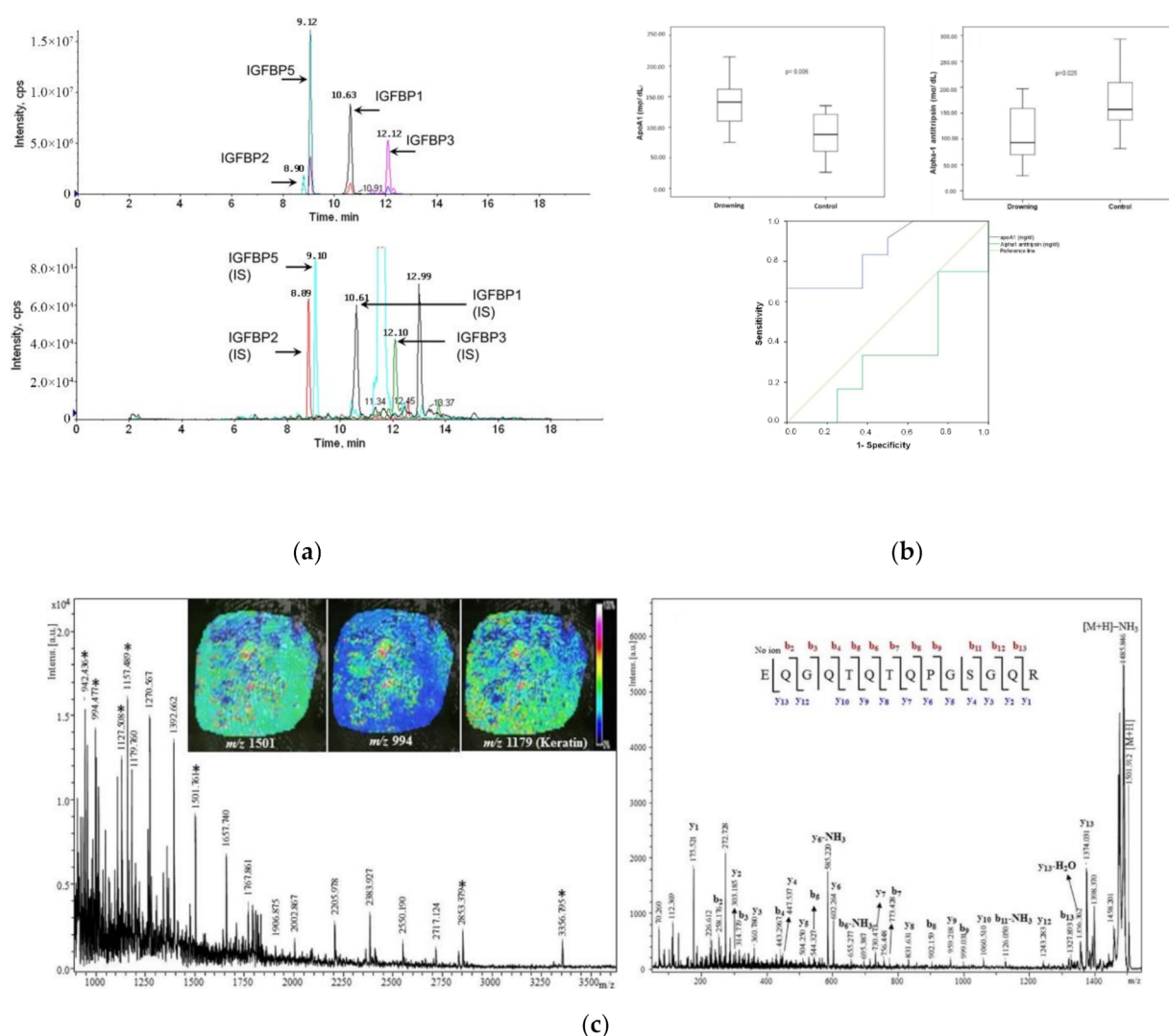


Figure 6. Other applications of forensic proteomics for human samples (a) LC–MS/MS chromatograms of IGFBP 1, 2, 3, and 5 and stable isotope-labeled peptides used as internal standards. Figure adapted from [48], Copyright (2019), with permission from Elsevier. (b) Plasma levels of apolipoprotein A1 (ApoA1) and alpha-1 antitrypsin in drowning and control samples (Student unpaired *t*-test) and Receiver Operating Characteristic (ROC) curves of ApoA1 and alpha-1 antitrypsin levels in the prediction of death by drowning. Figures were adapted from [50], under the terms of the Creative Commons Attribution License (CC BY 4.0) <https://creativecommons.org/licenses/by/4.0/> (accessed on 3 March 2021). (c) Matrix-assisted laser desorption ionization-time of flight (MALDI-ToF) imaging mass spectrometry (IMS) of a fingerprint contaminated with vaginal fluid (with the matched tryptic peptides of cornulin were asterisked) and MS/MS spectrum of cornulin tryptic peptide *m/z* 1501.91. Ion intensity maps of *m/z* 1501, 994 (peptides of cornulin), and 1179 (a peptide of human cytoskeletal keratin) were imaged. Figure reprinted by permission from Springer Nature, International Journal of Legal Medicine [35], Copyright (2017).

3.4.2. Fingernail

Fingernails can be used in forensics because body fluids and tissues may be trapped under the fingernails of individuals involved in violent contact during a crime. The traces under fingernails provide DNA profiles that indicate the origin of the traces. In addition, proteomic profiles can be obtained to determine the type of fluid or tissue in the traces. A recent study reported the detection of cornulin (a protein biomarker for vaginal fluid) up to 5 h and hemoglobin (a protein biomarker for blood) up to 18 h post-deposition in fingernails [34]. The authors proved that proteomic analysis can be complementarily performed using a genomics approach to provide forensic information from fingernail

debris. In the examined case, the DNA results showed that the suspect had touched the victim, whereas the proteomic results revealed that the touching involved the vagina.

3.4.3. Muscle

Mid-PMI (approximately 24–120 h PMI) can be estimated through the degradation of skeletal muscle proteins using gel electrophoresis, Western blotting, and casein zymography [169,170]. Proteomics is an alternative to these techniques. Choi et al. recently performed proteomic profiling to globally analyze changes in rat and mouse skeletal muscle proteomes at 0, 24, 48, 72, and 96 h PMI and selected two proteins that showed consistent degradation in both rat and mouse, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 1-alpha 2 (eEF1A2) [31]. In human autopsy cases, these proteins were feasible for PMI estimation, as validated through classical Western blot experiments. Thus, proteomic analysis of muscles could also be used in the forensic context. However, more studies in this field are required to determine and validate potential biomarkers prior to their practical application.

3.4.4. Brain and Cerebrospinal Fluid

Cerebrospinal fluid (CSF) can be used to determine the time of death by comparing the proteome profiles of ante-mortem and postmortem CSF. A previous study performed two-dimensional gel electrophoresis and MS. The authors found 14 proteins correlated with the postmortem interval (PMI), such as alpha-enolase, malate dehydrogenase, and peroxiredoxin 2 [32]. Broadbelt et al. performed a proteomic analysis of medullary tissues to investigate sudden infant death syndrome (SIDS) cases [49]. The authors revealed the reduction of 14-3-3 isoforms in the gigantocellularis of the medullary serotonin system, as well as levels of serotonin and tryptophan hydroxylase, suggesting that 14-3-3 isoforms could be used as diagnostic biomarkers for SIDS risk in living infants.

3.4.5. Blood

A recent study performed proteomic analysis of blood samples to identify potential protein biomarkers in the postmortem diagnosis of drowning [50]. When comparing the drowning group (16 samples) and control group (9 samples), apolipoprotein A1 (ApoA1) showed higher levels in the drowning group, whereas alpha-1 antitrypsin exhibited the opposite pattern (Figure 6b). Receiver operating characteristic (ROC) curves of ApoA1 and alpha-1 antitrypsin levels in the prediction of death by drowning were constructed (Figure 6b). Only ApoA1 levels reached statistical significance ($p < 0.01$), with an area under the curve of 0.85. A cut-off point of 100 mg/dL for ApoA1 was selected to determine drowning with the best specificity and sensitivity. This study is a pilot approach and should be further validated with a higher number of cases. The success of this preliminary study indicates the feasibility of proteomics in forensic diagnosis of drowning using blood samples.

3.4.6. Decomposition Fluid

A recent study reported a targeted proteomic analysis of pig decomposition fluid for the estimation of PMI [33]. A total of 29 peptides showing differences in the mean peak areas were identified by comparing samples collected in the early and late periods of decomposition in both summer and winter. Among them, eight peptides had significantly different fold changes between early and late PMI, including GHLDDPGAL (from hemoglobin subunit alpha), ESFGDLSNADAVMGNPVKV, FGDLSNADAVMGNPVK, GDL-SNADAVMGNPVKV (from hemoglobin subunit beta), KDLFDPIIQDR (from creatine kinase), IVGDDLTVTNPK (from beta-enolase), DLQHGSFL, and ILGQNGISDVVKV (from lactate dehydrogenase). Thus, PMI can also be predicted using proteomic analysis of the decomposition fluid. An untargeted method can also be used to obtain a full profile of protein degradation in post-mortem samples. It is also necessary to increase the sample size in future studies.

In addition to decomposition fluid, other types of human samples have been used for estimation of PMI, such as bone, muscle, brain, and cerebrospinal fluid (as discussed in Section 3.2, Section 3.4.3, and Section 3.4.4). Estimation of PMI (i.e., the time elapsed since death) is a crucial task in forensics to investigate unidentified human remains. A frequently used method is to assess gross morphological changes of the body visually. However, the rate of these changes is highly variable, and the reliability depends on the method and experience of investigators [171,172]. Biochemical techniques have emerged as precise and accurate methods to estimate PMI via analyses of proteins, metabolites, and RNA. These methods need to be validated for applications in forensic contexts [173–175]. Among the samples to be used, bone is a promising one for the development of PMI estimation methods with high accuracy, preciseness, and objectiveness. In bone, proteins are relatively stable and can be successfully extracted for proteomic analysis, whereas the analysis requires only a small amount of sample (microgram to sub-microgram of protein extract). Thus, it is possible to estimate late-PMI (e.g., >100 years) [41]. Other samples such as muscle, brain, cerebrospinal fluid, and decomposition fluid can be used for early and mid-PMI. Proteomic methods to estimate PMI have been developed and evaluated by many researchers. Some methods, as discussed above, were successfully applied in forensic contexts.

3.4.7. Fingerprint

In forensic science, fingerprints are valuable evidence for identifying the perpetrator of crime. The ridge pattern of the fingerprints could also contain additional chemical and biological evidence. Proteomics can be used to identify body fluids present in the fingerprints. In particular, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) IMS allows the direct mapping of foreign body fluids in fingerprints. Kamanna et al. applied MALDI-TOF IMS to discover traces of body fluids, such as blood and vaginal fluid [35]. Proteolysis of body fluids deposited in fingerprints can be implemented in approximately 12 h (conventional proteolysis) or 2 h using RapiGest-SF surfactant. Vaginal fluid deposited in fingerprints could be identified by spectra and maps related to the peptides of cornulin (m/z 1501 and 994) in MALDI-TOF IMS (Figure 6c). The ion intensity maps of m/z 1501 and 994 formed some ridges in the fingerprints, thus confirming the presence of vaginal fluid on the finger. The MS/MS spectrum of the precursor ion $[M + H]^+$ 1501.91 matched the peptide EQGQTQTQPGSGQR of cornulin with the identified ion fragments of 'b' and 'y' and loss of NH_3 (Figure 6c). Similarly, fingerprints containing blood showed ion intensity maps of m/z 1529 matched to the hemoglobin alpha-subunit, while m/z 1274 and 1314 matched to peptides of β -hemoglobin.

Oonk et al. performed proteomic profiling of fingerprints and identified 52 proteins [51]. Analysis of covariance (ANCOVA) was conducted for 31 proteins that were found in all sample pools to determine the effects of aging on protein abundance. The normalized abundances of 5 proteins were found to increase with age of the fingerprints, including keratin, type II cytoskeletal 1 (K2C1), Keratin, type II cytoskeletal 2 epidermal (K22E), Keratin, type I cytoskeletal 9 (K1C9), keratin, type I cytoskeletal 10 (K1C10), and dermcidin (DCD). The authors also investigated the effects of fingers contaminated with saliva, urine, and vaginal fluid on the proteome of fingerprints, which showed the presence of approximately 53%, 29%, and 44% of the proteome, respectively. Notably, all potential age-marker keratins were positively identified in the contaminated samples. In addition, unique proteins for the contaminated body fluids could be identified, such as mucin-5B and alpha-amylase-1, salivary acidic proline-rich phosphoprotein 1, carbonic anhydrase 6, cystatin-D for saliva, and uromodulin for urine, whereas no unique protein for vaginal fluid was found. The study, although in the preliminary stage, paves the way for the application of proteomics in determining age-markers of fingerprints.

3.4.8. Epidermal Corneocyte

Epidermal corneocytes may exist on fingerprints as trace evidence for forensic analysis. Borja et al. collected epidermal corneocytes from human skin with stripping tapes and used them for human identification [52]. They performed proteomic analysis and discovered 74 candidate GVPs, corresponding to 37 non-synonymous SNP loci from 31 genes. Thus, besides human hair and bone, epidermal corneocytes deposited on fingerprints are also valuable samples for human identification.

4. Challenges, Considerations, and Future of Forensic Proteomics

Various studies have reported potential applications of proteomics in forensic sciences. However, forensic proteomics may face several difficulties. Proteomic samples in other fields, such as microbiology, molecular biology, plant sciences, marine sciences, and food sciences, are generally clear for researchers (e.g., type of cell, tissue, or organism; species; sample matrices). In forensic proteomics, samples are sometimes unknown. In other fields of forensic science, such as identification of pathogenic species, sample preparation is generalized to the case of an uncharacterized sample. Taxonomy of the species can be narrowed down or determined using several methods [22,176]. For human-related samples, researchers have to identify which body fluids or tissues exist in the samples [45,149]. Another challenge in forensic proteomics is that samples may contain contaminants that are unknown to researchers. This causes many difficulties in defining which procedure to apply.

One of the biggest challenges in forensic proteomics is that the sample amount from trace evidence may be very low, making sample preparation difficult. That is the case of body fluids and tissues deposited in cloth, floor, the surface of solid materials (e.g., rock, plastic), under fingernails, or on skin. When the sample amount is low, low-abundance proteins cannot be identified. However, bottom-up proteomics has been developed to allow analysis of small sample amounts, such as a single hair of approximately 1 mm length [94], epidermal corneocytes from human skin [150], and debris deposited in fingernails [141]. Another challenge of forensic proteomics relates to sample preservation. In all cases, samples have to be collected and preserved in an appropriate manner to minimize sample degradation and contamination. Usually, sampling approaches include protocols for quality assurance and quality control. After collection, samples are tightly sealed and stored in short-term (at 4 °C) or long-term (at −20 °C or −80 °C), except for hair and nail [62].

The legal aspect is an important issue to consider in the development of proteomic methods for applications in forensics. The United States Supreme Court issued guidelines for the admissibility of scientific testimony in 1993, which is now called the Daubert standard [177]. One of the important requirements is that only scientific evidence obtained from scientific methods and principles is admissible, and the methods employed should have been tested, peer-reviewed, and widely accepted by the scientific community. In addition, methods and principles must be properly applied with suitable control samples, and error rates must be known or estimated [178]. Forensic proteomics, as a member of forensic science, should be developed and applied with sufficient consideration regarding legal issues. The proteomic method development process includes proof-of-concept, developmental verification and validation, and routine implementation (Figure 7). The proof-of-concept includes initial studies to develop protocols, determine sensitivity and specificity, and identify limitations. A standard operating protocol (SOP) constructed in this stage will be used in the second stage, developmental verification, and validation. This stage is performed with formal verification and validation experiments to determine the final SOP, identify operational limitations, and validate the methods in forensic samples with calibration and quality-control practices. All of these elements are essential for establishing reporting guidelines and obtaining certification from the International Organization for Standardization (ISO) or other accreditation prior to the application of the methods in routine operation [178]. In proteomics, a number of standard protocols and methodologies have been developed and widely applied [1,2,70,116]. They have also been used in forensic

science for various applications [12–18]. One of the appropriate estimations of error rates in proteomics is the false discovery rate (FDR). Thus, to be admissible in court, proteomics methods must undergo extensive method validation. New methods must be published for repetition by other groups and the acceptance of the scientific community [24].

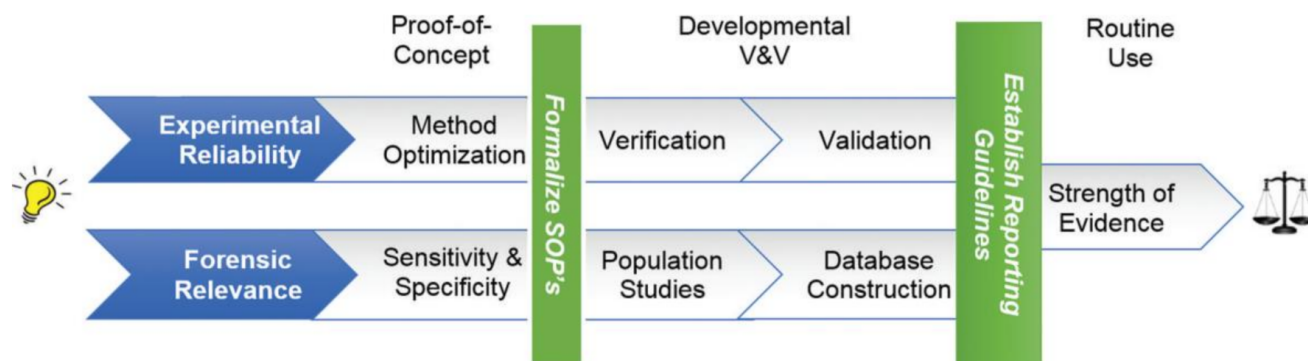


Figure 7. The forensic method development process. Reprinted with permission from [178]. Copyright (2019) American Chemical Society.

Proteomics is considered an interdisciplinary science because it depends on the development of analytical chemistry (peptide/protein identification and quantification), physical chemistry (sample separation), electrical engineering (mass spectrometry design), statistics, and bioinformatics (data analysis) [1,2]. The development and applications of proteomics have emerged as powerful tools in forensic science. Proteomics has attracted forensic scientists in recent years owing to its applicability to answer different forensic questions [4,11]. It can provide informative data that cannot be answered by DNA analysis. Although it is in its early stages, forensic proteomics will definitely continue to be studied and evaluated for applications in forensic contexts. Currently, forensic proteomics is developed for several applications, including human identification, PMI estimation, and identification of body fluids or tissues from unknown samples. Proteomic analysis of hair for human identification has been shown to be effective and robust [18,141]. Further studies will be conducted to validate the methods with a larger number of samples. Bone and some other samples (e.g., muscle, brain, cerebrospinal fluid, and decomposition fluid) can be used for the estimation of PMI. Among them, bone has been widely used, and a large number of protein biomarkers have been found [29]. Forensic proteomic studies of bone will be further carried out to identify new biomarkers and validate them. In addition, bone can be effective in estimating age-at-death via some protein biomarkers, such as AHSG [41]. Finally, the identification of body fluid (including their trace trapped in fingernail and on fingerprint) can be accomplished using the Van Steendam decision tree based on protein biomarkers of each fluid type [14]. More protein biomarkers will be continuously found to improve the identification workflow. In the case of body tissues, proteomic studies will focus on the identification of protein biomarkers for each tissue prior to applying them to distinguish tissue types [12]. As a new member of the forensic science family, proteomics requires considerable development time to be widely applied in this new field. In addition, efforts should be made to combine it with other well-established tools of forensic science.

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References

1. Tyers, M.; Mann, M. From genomics to proteomics. *Nature* **2003**, *422*, 193–197. [[CrossRef](#)] [[PubMed](#)]
2. Aebersold, R.; Mann, M. Mass spectrometry-based proteomics. *Nature* **2003**, *422*, 198–207. [[CrossRef](#)]
3. Pedrioli, P.G.A.; Eng, J.K.; Hubley, R.; Vogelzang, M.; Deutsch, E.W.; Raught, B.; Pratt, B.; Nilsson, E.; Angeletti, R.H.; Apweiler, R.; et al. A common open representation of mass spectrometry data and its application to proteomics research. *Nat. Biotechnol.* **2004**, *22*, 1459–1466. [[CrossRef](#)] [[PubMed](#)]
4. Merkley, E.D.; Wunschel, D.S.; Wahl, K.L.; Jarman, K.H. Applications and challenges of forensic proteomics. *Forensic Sci. Int.* **2019**, *297*, 350–363. [[CrossRef](#)] [[PubMed](#)]
5. Akçan, R.; Taştekin, B.; Yildirim, M.Ş.; Aydoğan, H.C.; Sağlam, N. Omics era in forensic medicine: Towards a new age. *Turk. J. Med. Sci.* **2020**, *50*, 1480–1490. [[CrossRef](#)]
6. Zhang, Z.; Wu, S.; Stenoien, D.L.; Paša-Tolić, L. High-throughput proteomics. *Annu. Rev. Anal. Chem.* **2014**, *7*, 427–454. [[CrossRef](#)] [[PubMed](#)]
7. Pedde, R.D.; Li, H.; Borchers, C.H.; Akbari, M. Microfluidic-mass spectrometry interfaces for translational proteomics. *Trends Biotechnol.* **2017**, *35*, 954–970. [[CrossRef](#)]
8. Kuras, M.; Betancourt, L.H.; Rezeli, M.; Rodriguez, J.; Szasz, M.; Zhou, Q.; Miliotis, T.; Andersson, R.; Marko-Varga, G. Assessing automated sample preparation technologies for high-throughput proteomics of frozen well characterized tissues from Swedish biobanks. *J. Proteome Res.* **2019**, *18*, 548–556. [[CrossRef](#)]
9. Fu, Q.; Kowalski, M.P.; Mastali, M.; Parker, S.J.; Sobhani, K.; van den Broek, I.; Hunter, C.L.; Van Eyk, J.E. Highly reproducible automated proteomics sample preparation workflow for quantitative mass spectrometry. *J. Proteome Res.* **2018**, *17*, 420–428. [[CrossRef](#)]
10. Alexovič, M.; Urban, P.L.; Tabani, H.; Sabo, J. Recent advances in robotic protein sample preparation for clinical analysis and other biomedical applications. *Clin. Chim. Acta* **2020**, *507*, 104–116. [[CrossRef](#)]
11. Moini, M. Applications of liquid-based separation in conjunction with mass spectrometry to the analysis of forensic evidence. *Electrophoresis* **2018**, *39*, 1249–1275. [[CrossRef](#)] [[PubMed](#)]
12. Legg, K.M.; Powell, R.; Reisdorph, N.; Reisdorph, R.; Danielson, P.B. Verification of protein biomarker specificity for the identification of biological stains by quadrupole time-of-flight mass spectrometry. *Electrophoresis* **2017**, *38*, 833–845. [[CrossRef](#)] [[PubMed](#)]
13. Kamanna, S.; Henry, J.; Voelcker, N.; Linacre, A.; Kirkbride, K.P. ‘Bottom-up’ in situ proteomic differentiation of human and non-human haemoglobins for forensic purposes by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2017**, *31*, 1927–1937. [[CrossRef](#)] [[PubMed](#)]
14. Van Steendam, K.; De Ceuleneer, M.; Dhaenens, M.; Van Hoofstat, D.; Deforce, D. Mass spectrometry-based proteomics as a tool to identify biological matrices in forensic science. *Int. J. Legal Med.* **2013**, *127*, 287–298. [[CrossRef](#)] [[PubMed](#)]
15. Abd El-Aziz, T.M.; Soares, A.G.; Stockand, J.D. Advances in venomomics: Modern separation techniques and mass spectrometry. *J. Chromatogr. B* **2020**, *1160*, 122352. [[CrossRef](#)] [[PubMed](#)]
16. Sauvage, S.; Hardouin, J. Exoproteomics for better understanding pseudomonas aeruginosa virulence. *Toxins* **2020**, *12*, 571. [[CrossRef](#)] [[PubMed](#)]
17. Adeola, H.A.; Van Wyk, J.C.; Arowolo, A.; Ngwanya, R.M.; Mkentane, K.; Khumalo, N.P. Emerging diagnostic and therapeutic potentials of human hair proteomics. *Proteom. Clin. Appl.* **2018**, *12*, 1700048. [[CrossRef](#)] [[PubMed](#)]
18. Parker, G.J.; Leppert, T.; Anex, D.S.; Hilmer, J.K.; Matsunami, N.; Baird, L.; Stevens, J.; Parsawar, K.; Durbin-Johnson, B.P.; Rocke, D.M.; et al. Demonstration of protein-based human identification using the hair shaft proteome. *PLoS ONE* **2016**, *11*, e0160653. [[CrossRef](#)]
19. Thevis, M.; Thomas, A.; Geyer, H.; Schänzer, W. Mass spectrometric characterization of a biotechnologically produced full-length mechano growth factor (MGF) relevant for doping controls. *Growth Horm. IGF Res.* **2014**, *24*, 276–280. [[CrossRef](#)]
20. Thevis, M.; Kuuranne, T.; Geyer, H. Annual banned-substance review—Analytical approaches in human sports drug testing. *Drug Test. Anal.* **2020**, *12*, 7–26. [[CrossRef](#)]
21. Leiser, O.P.; Blackburn, J.K.; Hadfield, T.L.; Kreuzer, H.W.; Wunschel, D.S.; Bruckner-Lea, C.J. Laboratory strains of *Bacillus anthracis* exhibit pervasive alteration in expression of proteins related to sporulation under laboratory conditions relative to genetically related wild strains. *PLoS ONE* **2018**, *13*, e0209120. [[CrossRef](#)]
22. Boulund, F.; Karlsson, R.; Gonzales-Siles, L.; Johnning, A.; Karami, N.; AL-Bayati, O.; Ahren, C.; Moore, E.R.B.; Kristiansson, E. TCUP: Typing and characterization of bacteria using bottom-up tandem mass spectrometry proteomics. *Mol. Cell. Proteom.* **2017**. [[CrossRef](#)]

23. Merkley, E.D.; Kaiser, B.L.D.; Kreuzer, H. A proteomics tutorial. In *Applications in Forensic Proteomics: Protein Identification and Profiling*; American Chemical Society: Washington, DC, USA, 2019; Volume 1339, pp. 9–28.
24. Nicora, C.; Gritsenko, M.; Lipton, A.; Wahl, K.L.; Burnum-Johnson, K.E. Proteomic sample preparation techniques: Toward forensic proteomic applications. In *Applications in Forensic Proteomics: Protein Identification and Profiling*; American Chemical Society: Washington, DC, USA, 2019; Volume 1339, pp. 29–46.
25. Cole, L.M.; Clench, M.R.; Francese, S. Sample treatment for tissue proteomics in cancer, toxicology, and forensics. In *Emerging Sample Treatments in Proteomics*; Capelo-Martínez, J.-L., Ed.; Springer International Publishing: Cham, Germany, 2019; pp. 77–123. [\[CrossRef\]](#)
26. Mohamed Nasir, N.; Hiji, J.; Jayapalan, J.J.; Hashim, O.H. Potential use of human hair shaft keratin peptide signatures to distinguish gender and ethnicity. *PeerJ* **2020**, *8*, e8248. [\[CrossRef\]](#)
27. Chu, F.; Mason, K.E.; Anex, D.S.; Jones, A.D.; Hart, B.R. Hair proteome variation at different body locations on genetically variant peptide detection for protein-based human identification. *Sci. Rep.* **2019**, *9*, 7641. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Oran, P.E.; Jarvis, J.W.; Borges, C.R.; Sherma, N.D.; Nelson, R.W. Mass spectrometric immunoassay of intact insulin and related variants for population proteomics studies. *Proteom. Clin. Appl.* **2011**, *5*, 454–459. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Procopio, N.; Williams, A.; Chamberlain, A.T.; Buckley, M. Forensic proteomics for the evaluation of the post-mortem decay in bones. *J. Proteom.* **2018**, *177*, 21–30. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Prieto-Bonete, G.; Pérez-Cárceles, M.D.; Maurandi-López, A.; Pérez-Martínez, C.; Luna, A. Association between protein profile and postmortem interval in human bone remains. *J. Proteom.* **2019**, *192*, 54–63. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Choi, K.-M.; Zissler, A.; Kim, E.; Ehrenfellner, B.; Cho, E.; Lee, S.-i.; Steinbacher, P.; Yun, K.N.; Shin, J.H.; Kim, J.Y.; et al. Postmortem proteomics to discover biomarkers for forensic PMI estimation. *Int. J. Legal Med.* **2019**, *133*, 899–908. [\[CrossRef\]](#)
32. Finehout, E.J.; Franck, Z.; Relkin, N.; Lee, K.H. Proteomic analysis of cerebrospinal fluid changes related to postmortem interval. *Clin. Chem.* **2006**, *52*, 1906–1913. [\[CrossRef\]](#)
33. Nolan, A.-N.D.; Maker, G.; Mead, R.J.; Bringans, S.; Speers, S.J. Peptide analysis of mammalian decomposition fluid in relation to the post-mortem interval. *Forensic Sci. Int.* **2020**, *311*, 110269. [\[CrossRef\]](#)
34. Kamanna, S.; Henry, J.; Voelcker, N.; Linacre, A.; Paul Kirkbride, K. A complementary forensic ‘proteo-genomic’ approach for the direct identification of biological fluid traces under fingernails. *Anal. Bioanal. Chem.* **2018**, *410*, 6165–6175. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Kamanna, S.; Henry, J.; Voelcker, N.H.; Linacre, A.; Paul Kirkbride, K. A mass spectrometry-based forensic toolbox for imaging and detecting biological fluid evidence in finger marks and fingernail scrapings. *Int. J. Legal Med.* **2017**, *131*, 1413–1422. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Laatsch, C.N.; Durbin-Johnson, B.P.; Rocke, D.M.; Mukwana, S.; Newland, A.B.; Flagler, M.J.; Davis, M.G.; Eigenheer, R.A.; Phinney, B.S.; Rice, R.H. Human hair shaft proteomic profiling: Individual differences, site specificity and cuticle analysis. *PeerJ* **2014**, *2*, e506. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Goecker, Z.C.; Salemi, M.R.; Karim, N.; Phinney, B.S.; Rice, R.H.; Parker, G.J. Optimal processing for proteomic genotyping of single human hairs. *Forensic Sci. Int. Genet.* **2020**, *47*, 102314. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Mason, K.E.; Paul, P.H.; Chu, F.; Anex, D.S.; Hart, B.R. Development of a Protein-based Human Identification Capability from a Single Hair. *J. Forensic Sci.* **2019**, *64*, 1152–1159. [\[CrossRef\]](#)
39. Milan, J.A.; Wu, P.-W.; Salemi, M.R.; Durbin-Johnson, B.P.; Rocke, D.M.; Phinney, B.S.; Rice, R.H.; Parker, G.J. Comparison of protein expression levels and proteomically-inferred genotypes using human hair from different body sites. *Forensic Sci. Int. Genet.* **2019**, *41*, 19–23. [\[CrossRef\]](#)
40. Procopio, N.; Chamberlain, A.T.; Buckley, M. Intra- and interskeletal proteome variations in fresh and buried bones. *J. Proteome Res.* **2017**, *16*, 2016–2029. [\[CrossRef\]](#)
41. Procopio, N.; Chamberlain, A.T.; Buckley, M. Exploring biological and geological age-related changes through variations in intra- and intertooth proteomes of ancient dentine. *J. Proteome Res.* **2018**, *17*, 1000–1013. [\[CrossRef\]](#)
42. Sawafuji, R.; Cappellini, E.; Nagaoka, T.; Fotakis, A.K.; Jersie-Christensen, R.R.; Olsen, J.V.; Hirata, K.; Ueda, S. Proteomic profiling of archaeological human bone. *R. Soc. Open Sci.* **2017**, *4*, 161004. [\[CrossRef\]](#)
43. Mason, K.E.; Anex, D.; Grey, T.; Hart, B.; Parker, G. Protein-based forensic identification using genetically variant peptides in human bone. *Forensic. Sci. Int.* **2018**, *288*, 89–96. [\[CrossRef\]](#)
44. Kennedy, K.; Heaton, C.; Langenburg, G.; Cole, L.; Clark, T.; Clench, M.R.; Sears, V.; Sealey, M.; McColm, R.; Francese, S. Pre-validation of a MALDI MS proteomics-based method for the reliable detection of blood and blood provenance. *Sci. Rep.* **2020**, *10*, 17087. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Dammeier, S.; Nahnsen, S.; Veit, J.; Wehner, F.; Ueffing, M.; Kohlbacher, O. Mass-spectrometry-based proteomics reveals organ-specific expression patterns to be used as forensic evidence. *J. Proteome Res.* **2016**, *15*, 182–192. [\[CrossRef\]](#)
46. Di Meo, A.; Sohaei, D.; Batruch, I.; Alexandrou, P.; Prassas, I.; Diamandis, E.P. Proteomic Profiling of the Human Tissue and Biological Fluid Proteome. *J. Proteome Res.* **2020**. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Peterman, S.; Niederkofler, E.E.; Phillips, D.A.; Krastins, B.; Kiernan, U.A.; Tubbs, K.A.; Nedelkov, D.; Prakash, A.; Vogelsang, M.S.; Schoeder, T.; et al. An automated, high-throughput method for targeted quantification of intact insulin and its therapeutic analogs in human serum or plasma coupling mass spectrometric immunoassay with high resolution and accurate mass detection (MSIA-HR/AM). *Proteomics* **2014**, *14*, 1445–1456. [\[CrossRef\]](#) [\[PubMed\]](#)

48. Liu, N.; Tengstrand, E.; Boernsen, K.O.; Bek, S.; Hsieh, F. Validation of a multiplexed LC–MS/MS clinical assay to quantify insulin-like growth factor-binding proteins in human serum and its application in a clinical study. *Toxicol. Appl. Pharmacol.* **2019**, *371*, 74–83. [\[CrossRef\]](#)
49. Broadbelt, K.G.; Rivera, K.D.; Paterson, D.S.; Duncan, J.R.; Trachtenberg, F.L.; Paulo, J.A.; Stapels, M.D.; Borenstein, N.S.; Belliveau, R.A.; Haas, E.A.; et al. Brainstem deficiency of the 14-3-3 regulator of serotonin synthesis: A proteomics analysis in the sudden infant death syndrome. *Mol. Cell. Proteom.* **2012**, *11*. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Hernández-Romero, D.; Sánchez-Rodríguez, E.; Osuna, E.; Sibón, A.; Martínez-Villanueva, M.; Noguera-Velasco, J.A.; Pérez-Cárceles, M.D. Proteomics in deaths by drowning: Diagnostic efficacy of apolipoprotein a1 and α -1 antitrypsin, pilot study. *Diagnostics* **2020**, *10*, 747. [\[CrossRef\]](#)
51. Oonk, S.; Schuurmans, T.; Pabst, M.; de Smet, L.C.P.M.; de Puit, M. Proteomics as a new tool to study fingermark ageing in forensics. *Sci. Rep.* **2018**, *8*, 16425. [\[CrossRef\]](#)
52. Borja, T.; Karim, N.; Goecker, Z.; Salemi, M.; Phinney, B.; Naeem, M.; Rice, R.; Parker, G. Proteomic genotyping of fingermark donors with genetically variant peptides. *Forens. Sci. Int. Genet.* **2019**, *42*, 21–30. [\[CrossRef\]](#)
53. Cai, W.; Tucholski, T.M.; Gregorich, Z.R.; Ge, Y. Top-down proteomics: Technology advancements and applications to heart diseases. *Expert Rev. Proteom.* **2016**, *13*, 717–730. [\[CrossRef\]](#)
54. Gregorich, Z.R.; Ge, Y. Top-down proteomics in health and disease: Challenges and opportunities. *Proteomics* **2014**, *14*, 1195–1210. [\[CrossRef\]](#)
55. Duong, V.-A.; Park, J.-M.; Lee, H. Review of three-dimensional liquid chromatography platforms for bottom-up proteomics. *Int. J. Mol. Sci.* **2020**, *21*, 1524. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Pandeswari, P.B.; Sabareesh, V. Middle-down approach: A choice to sequence and characterize proteins/proteomes by mass spectrometry. *RSC Adv.* **2019**, *9*, 313–344. [\[CrossRef\]](#)
57. Sidoli, S.; Garcia, B.A. Middle-down proteomics: A still unexploited resource for chromatin biology. *Expert Rev. Proteom.* **2017**, *14*, 617–626. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Zhou, H.; Ning, Z.; Starr, A.E.; Abu-Farha, M.; Figeys, D. Advancements in top-down proteomics. *Anal. Chem.* **2012**, *84*, 720–734. [\[CrossRef\]](#)
59. Chen, B.; Brown, K.A.; Lin, Z.; Ge, Y. Top-down proteomics: Ready for prime time? *Anal. Chem.* **2018**, *90*, 110–127. [\[CrossRef\]](#)
60. Dinis-Oliveira, R.J.; Vieira, D.N.; Magalhães, T. Guidelines for collection of biological samples for clinical and forensic toxicological analysis. *Forensic Sci. Res.* **2016**, *1*, 42–51. [\[CrossRef\]](#)
61. Budowle, B.; Schutzer, S.E.; Burans, J.P.; Beecher, D.J.; Cebula, T.A.; Chakraborty, R.; Cobb, W.T.; Fletcher, J.; Hale, M.L.; Harris, R.B.; et al. Quality sample collection, handling, and preservation for an effective microbial forensics program. *Appl. Environ. Microbiol.* **2006**, *72*, 6431–6438. [\[CrossRef\]](#)
62. Dinis-Oliveira, R.J.; Carvalho, F.; Duarte, J.A.; Remião, F.; Marques, A.; Santos, A.; Magalhães, T. Collection of biological samples in forensic toxicology. *Toxicol. Mech. Methods* **2010**, *20*, 363–414. [\[CrossRef\]](#)
63. Koh, K.; Park, M.; Bae, E.S.; Duong, V.-A.; Park, J.-M.; Lee, H.; Lew, H. UBA2 activates Wnt/ β -catenin signaling pathway during protection of R28 retinal precursor cells from hypoxia by extracellular vesicles derived from placental mesenchymal stem cells. *Stem Cell Res. Ther.* **2020**, *11*, 428. [\[CrossRef\]](#)
64. Van-An, D.; Jeeyun, A.; Na-Young, H.; Jong-Moon, P.; Jeong-Hun, M.; Tae Wan, K.; Hookeun, L. Proteomic Analysis of the vitreous body in proliferative and non-proliferative diabetic retinopathy. *Curr. Proteom.* **2020**, *17*, 1. [\[CrossRef\]](#)
65. Kwon, D.; Park, J.-M.; Duong, V.-A.; Hong, S.-J.; Cho, B.-K.; Lee, C.-G.; Choi, H.-K.; Kim, D.-M.; Lee, H. Comparative proteomic profiling of marine and freshwater synechocystis strains using liquid chromatography-tandem mass spectrometry. *J. Marine Sci. Eng.* **2020**, *8*, 790. [\[CrossRef\]](#)
66. Procopio, N.; Buckley, M. Minimizing laboratory-induced decay in bone proteomics. *J. Proteome Res.* **2017**, *16*, 447–458. [\[CrossRef\]](#)
67. Azzam, S.; Broadwater, L.; Li, S.; Freeman, E.J.; McDonough, J.; Gregory, R.B. A SELDI mass spectrometry study of experimental autoimmune encephalomyelitis: Sample preparation, reproducibility, and differential protein expression patterns. *Proteome Sci.* **2013**, *11*, 19. [\[CrossRef\]](#)
68. Toni, M.; Angiulli, E.; Miccoli, G.; Cioni, C.; Alleva, E.; Frabetti, F.; Pizzetti, F.; Grassi Scalvini, F.; Nonnis, S.; Negri, A.; et al. Environmental temperature variation affects brain protein expression and cognitive abilities in adult zebrafish (*Danio rerio*): A proteomic and behavioural study. *J. Proteom.* **2019**, *204*, 103396. [\[CrossRef\]](#)
69. Yagi, R.; Masuda, T.; Ogata, S.; Mori, A.; Ito, S.; Ohtsuki, S. Proteomic evaluation of plasma membrane fraction prepared from a mouse liver and kidney using a bead homogenizer: Enrichment of drug-related transporter proteins. *Mol. Pharm.* **2020**, *17*, 4101–4113. [\[CrossRef\]](#)
70. Mertins, P.; Tang, L.C.; Krug, K.; Clark, D.J.; Gritsenko, M.A.; Chen, L.; Clauser, K.R.; Clauss, T.R.; Shah, P.; Gillette, M.A.; et al. Reproducible workflow for multiplexed deep-scale proteome and phosphoproteome analysis of tumor tissues by liquid chromatography–mass spectrometry. *Nat. Protoc.* **2018**, *13*, 1632–1661. [\[CrossRef\]](#)
71. Guyuron, B.; Yohannes, E.; Miller, R.; Chim, H.; Reed, D.; Chance, M.R. Electron microscopic and proteomic comparison of terminal branches of the trigeminal nerve in patients with and without migraine headaches. *Plast. Reconstr. Surg.* **2014**, *134*, 796e–805e. [\[CrossRef\]](#)

72. Valdés-López, O.; Batek, J.; Gomez-Hernandez, N.; Nguyen, C.T.; Isidra-Arellano, M.C.; Zhang, N.; Joshi, T.; Xu, D.; Hixson, K.K.; Weitz, K.K.; et al. Soybean roots grown under heat stress show global changes in their transcriptional and proteomic profiles. *Front. Plant Sci.* **2016**, *7*. [[CrossRef](#)]
73. Isaacson, T.; Damasceno, C.M.B.; Saravanan, R.S.; He, Y.; Catalá, C.; Saladié, M.; Rose, J.K.C. Sample extraction techniques for enhanced proteomic analysis of plant tissues. *Nat. Protoc.* **2006**, *1*, 769–774. [[CrossRef](#)]
74. Yun, G.; Park, J.-M.; Duong, V.-A.; Mok, J.-H.; Jeon, J.; Nam, O.; Lee, J.; Jin, E.; Lee, H. Proteomic profiling of *emiliana huxleyi* using a three-dimensional separation method combined with tandem mass spectrometry. *Molecules* **2020**, *25*, 3028. [[CrossRef](#)]
75. Santa, C.; Anjo, S.I.; Manadas, B. Protein precipitation of diluted samples in SDS-containing buffer with acetone leads to higher protein recovery and reproducibility in comparison with TCA/acetone approach. *Proteomics* **2016**, *16*, 1847–1851. [[CrossRef](#)]
76. Evans, D.R.H.; Romero, J.K.; Westoby, M. Chapter 9 concentration of proteins and removal of solutes. In *Methods in Enzymology*; Burgess, R.R., Deutscher, M.P., Eds.; Academic Press: Cambridge, MA, USA, 2009; Volume 463, pp. 97–120.
77. Tu, C.; Rudnick, P.A.; Martinez, M.Y.; Cheek, K.L.; Stein, S.E.; Slebos, R.J.C.; Liebler, D.C. Depletion of abundant plasma proteins and limitations of plasma proteomics. *J. Proteome Res.* **2010**, *9*, 4982–4991. [[CrossRef](#)]
78. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [[CrossRef](#)]
79. Smith, P.K.; Krohn, R.I.; Hermanson, G.T.; Mallia, A.K.; Gartner, F.H.; Provenzano, M.D.; Fujimoto, E.K.; Goeke, N.M.; Olson, B.J.; Klenk, D.C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76–85. [[CrossRef](#)]
80. Legler, G.; Müller-Platz, C.M.; Mentges-Hettkamp, M.; Pflieger, G.; Jülich, E. On the chemical basis of the Lowry protein determination. *Anal. Biochem.* **1985**, *150*, 278–287. [[CrossRef](#)]
81. Donnell, A.M.; Lewis, S.; Abraham, S.; Subramanian, K.; Figueroa, J.L.; Deepe, G.S.; Vonderheide, A.P. Investigation of an optimal cell lysis method for the study of the zinc metalloproteome of *Histoplasma capsulatum*. *Anal. Bioanal. Chem.* **2017**, *409*, 6163–6172. [[CrossRef](#)]
82. Oswald, E.S.; Brown, L.M.; Bulinski, J.C.; Hung, C.T. Label-free protein profiling of adipose-derived human stem cells under hyperosmotic treatment. *J. Proteome Res.* **2011**, *10*, 3050–3059. [[CrossRef](#)]
83. Yu, Y.-Q.; Gilar, M.; Lee, P.J.; Bouvier, E.S.P.; Gebler, J.C. Enzyme-friendly, mass spectrometry-compatible surfactant for in-solution enzymatic digestion of proteins. *Anal. Chem.* **2003**, *75*, 6023–6028. [[CrossRef](#)]
84. Shevchenko, A.; Tomas, H.; Havli, J.; Olsen, J.V.; Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **2006**, *1*, 2856–2860. [[CrossRef](#)]
85. Hughes, C.S.; Foehr, S.; Garfield, D.A.; Furlong, E.E.; Steinmetz, L.M.; Krijgsveld, J. Ultrasensitive proteome analysis using paramagnetic bead technology. *Mol. Syst. Biol.* **2014**, *10*, 757. [[CrossRef](#)]
86. Hughes, C.S.; Moggridge, S.; Müller, T.; Sorensen, P.H.; Morin, G.B.; Krijgsveld, J. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. *Nat. Protoc.* **2019**, *14*, 68–85. [[CrossRef](#)]
87. Raghunathan, R.; Sethi, M.K.; Zaia, J. On-slide tissue digestion for mass spectrometry based glycomic and proteomic profiling. *MethodsX* **2019**, *6*, 2329–2347. [[CrossRef](#)]
88. Judd, A.M.; Gutierrez, D.B.; Moore, J.L.; Patterson, N.H.; Yang, J.; Romer, C.E.; Norris, J.L.; Caprioli, R.M. A recommended and verified procedure for in situ tryptic digestion of formalin-fixed paraffin-embedded tissues for analysis by matrix-assisted laser desorption/ionization imaging mass spectrometry. *J. Mass Spectrom.* **2019**, *54*, 716–727. [[CrossRef](#)]
89. Wiśniewski, J.R. Filter aided sample preparation—A tutorial. *Anal. Chim. Acta* **2019**. [[CrossRef](#)]
90. Wiśniewski, J.R.; Zougman, A.; Nagaraj, N.; Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* **2009**, *6*, 359–362. [[CrossRef](#)]
91. Davalieva, K.; Kiprijanovska, S.; Dimovski, A.; Rosoklija, G.; Dwork, A.J. Comparative evaluation of two methods for LC-MS/MS proteomic analysis of formalin fixed and paraffin embedded tissues. *J. Proteom.* **2021**, *235*, 104117. [[CrossRef](#)]
92. Ludwig, K.R.; Schroll, M.M.; Hummon, A.B. Comparison of In-Solution, FASP, and S-trap based digestion methods for bottom-up proteomic studies. *J. Proteome Res.* **2018**, *17*, 2480–2490. [[CrossRef](#)]
93. HaileMariam, M.; Egue, R.V.; Singh, H.; Bekele, S.; Ameni, G.; Pieper, R.; Yu, Y. S-Trap, an ultrafast sample-preparation approach for shotgun proteomics. *J. Proteome Res.* **2018**, *17*, 2917–2924. [[CrossRef](#)]
94. Elinger, D.; Gabashvili, A.; Levin, Y. suspension trapping (s-trap) is compatible with typical protein extraction buffers and detergents for bottom-up proteomics. *J. Proteome Res.* **2019**, *18*, 1441–1445. [[CrossRef](#)]
95. Olsen, J.V.; Ong, S.-E.; Mann, M. Trypsin cleaves exclusively c-terminal to arginine and lysine residues. *Mol. Cell. Proteom.* **2004**, *3*, 608–614. [[CrossRef](#)]
96. Schmelter, C.; Funke, S.; Treml, J.; Beschnitt, A.; Perumal, N.; Manicam, C.; Pfeiffer, N.; Grus, F.H. Comparison of two solid-phase extraction (SPE) methods for the identification and quantification of porcine retinal protein markers by LC-MS/MS. *Int. J. Mol. Sci.* **2018**, *19*, 3847. [[CrossRef](#)]
97. Shen, Y.; Tolić, N.; Masselon, C.; Paša-Tolić, L.; Camp, D.G.; Hixson, K.K.; Zhao, R.; Anderson, G.A.; Smith, R.D. Ultrasensitive proteomics using high-efficiency on-line micro-SPE-NanoLC-NanoESI MS and MS/MS. *Anal. Chem.* **2004**, *76*, 144–154. [[CrossRef](#)] [[PubMed](#)]
98. Bladergroen, M.R.; van der Burgt, Y.E.M. Solid-phase extraction strategies to surmount body fluid sample complexity in high-throughput mass spectrometry-based proteomics. *J. Anal. Methods Chem.* **2015**, *2015*, 250131. [[CrossRef](#)]

99. Chen, D.; Shen, X.; Sun, L. Strong cation exchange-reversed phase liquid chromatography-capillary zone electrophoresis-tandem mass spectrometry platform with high peak capacity for deep bottom-up proteomics. *Anal. Chim. Acta* **2018**, *1012*, 1–9. [[CrossRef](#)] [[PubMed](#)]
100. Boichenko, A.P.; Govorukhina, N.; van der Zee, A.G.J.; Bischoff, R. Multidimensional separation of tryptic peptides from human serum proteins using reversed-phase, strong cation exchange, weak anion exchange, and fused-core fluorinated stationary phases. *J. Sep. Sci.* **2013**, *36*, 3463–3470. [[CrossRef](#)]
101. Garbis, S.D.; Roumeliotis, T.I.; Tyritzis, S.I.; Zorpas, K.M.; Pavlakis, K.; Constantinides, C.A. A Novel multidimensional protein identification technology approach combining protein size exclusion prefractionation, peptide zwitterion–ion hydrophilic interaction chromatography, and nano-ultraperformance RP chromatography/nESI-MS2 for the in-depth analysis of the serum proteome and phosphoproteome: Application to clinical sera derived from humans with benign prostate hyperplasia. *Anal. Chem.* **2011**, *83*, 708–718. [[CrossRef](#)] [[PubMed](#)]
102. Spicer, V.; Ezzati, P.; Neustaeter, H.; Beavis, R.C.; Wilkins, J.A.; Krokhn, O.V. 3D HPLC-MS with reversed-phase separation functionality in all three dimensions for large-scale bottom-up proteomics and peptide retention data collection. *Anal. Chem.* **2016**, *88*, 2847–2855. [[CrossRef](#)]
103. Zhao, P.; Schulz, T.C.; Sherrer, E.S.; Weatherly, D.B.; Robins, A.J.; Wells, L. The human embryonic stem cell proteome revealed by multidimensional fractionation followed by tandem mass spectrometry. *Proteomics* **2015**, *15*, 554–566. [[CrossRef](#)]
104. Bantscheff, M.; Lemeer, S.; Savitski, M.M.; Kuster, B. Quantitative mass spectrometry in proteomics: Critical review update from 2007 to the present. *Anal. Bioanal. Chem.* **2012**, *404*, 939–965. [[CrossRef](#)]
105. Vitzthum, F.; Behrens, F.; Anderson, N.L.; Shaw, J.H. Proteomics: From basic research to diagnostic application. a review of requirements & needs. *J. Proteome Res.* **2005**, *4*, 1086–1097. [[CrossRef](#)]
106. Vidova, V.; Spacil, Z. A review on mass spectrometry-based quantitative proteomics: Targeted and data independent acquisition. *Anal. Chim. Acta* **2017**, *964*, 7–23. [[CrossRef](#)]
107. Eng, J.K.; Searle, B.C.; Clauser, K.R.; Tabb, D.L. A Face in the crowd: Recognizing peptides through database search. *Mol. Cell. Proteom.* **2011**, *10*. [[CrossRef](#)]
108. Eng, J.K.; McCormack, A.L.; Yates, J.R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 976–989. [[CrossRef](#)]
109. Perkins, D.N.; Pappin, D.J.C.; Creasy, D.M.; Cottrell, J.S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20*, 3551–3567. [[CrossRef](#)]
110. Craig, R.; Beavis, R.C. TANDEM: Matching proteins with tandem mass spectra. *Bioinformatics* **2004**, *20*, 1466–1467. [[CrossRef](#)]
111. Cox, J.; Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **2008**, *26*, 1367–1372. [[CrossRef](#)] [[PubMed](#)]
112. Zhang, J.; Xin, L.; Shan, B.; Chen, W.; Xie, M.; Yuen, D.; Zhang, W.; Zhang, Z.; Lajoie, G.A.; Ma, B. PEAKS DB: De Novo sequencing assisted database search for sensitive and accurate peptide identification. *Mol. Cell. Proteom.* **2012**, *11*. [[CrossRef](#)] [[PubMed](#)]
113. Yuan, J.; Zhang, R.; Yang, Z.; Lee, J.; Liu, Y.; Tian, J.; Qin, X.; Ren, Z.; Ding, H.; Chen, Q.; et al. Comparative Effectiveness and safety of oral phosphodiesterase type 5 inhibitors for erectile dysfunction: A Systematic review and network meta-analysis. *Eur. Urol.* **2013**, *63*, 902–912. [[CrossRef](#)] [[PubMed](#)]
114. Kong, A.T.; Leprevost, F.V.; Avtonomov, D.M.; Mellacheruvu, D.; Nesvizhskii, A.I. MSFragger: Ultrafast and comprehensive peptide identification in mass spectrometry-based proteomics. *Nat. Methods* **2017**, *14*, 513–520. [[CrossRef](#)]
115. Keller, A.; Nesvizhskii, A.I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **2002**, *74*, 5383–5392. [[CrossRef](#)]
116. Käll, L.; Canterbury, J.D.; Weston, J.; Noble, W.S.; MacCoss, M.J. Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat. Methods* **2007**, *4*, 923–925. [[CrossRef](#)]
117. Ma, Z.-Q.; Dasari, S.; Chambers, M.C.; Litton, M.D.; Sobecki, S.M.; Zimmerman, L.J.; Halvey, P.J.; Schilling, B.; Drake, P.M.; Gibson, B.W.; et al. IDPicker 2.0: Improved protein assembly with high discrimination peptide identification filtering. *J. Proteome Res.* **2009**, *8*, 3872–3881. [[CrossRef](#)]
118. Griss, J. Spectral library searching in proteomics. *Proteomics* **2016**, *16*, 729–740. [[CrossRef](#)] [[PubMed](#)]
119. Lam, H.; Deutsch, E.W.; Eddes, J.S.; Eng, J.K.; King, N.; Stein, S.E.; Aebersold, R. Development and validation of a spectral library searching method for peptide identification from MS/MS. *Proteomics* **2007**, *7*, 655–667. [[CrossRef](#)] [[PubMed](#)]
120. Craig, R.; Cortens, J.C.; Fenyo, D.; Beavis, R.C. Using annotated peptide mass spectrum libraries for protein identification. *J. Proteome Res.* **2006**, *5*, 1843–1849. [[CrossRef](#)] [[PubMed](#)]
121. Ahrné, E.; Nikitin, F.; Lisacek, F.; Müller, M. QuickMod: A tool for open modification spectrum library searches. *J. Proteome Res.* **2011**, *10*, 2913–2921. [[CrossRef](#)]
122. Dasari, S.; Chambers, M.C.; Martinez, M.A.; Carpenter, K.L.; Ham, A.-J.L.; Vega-Montoto, L.J.; Tabb, D.L. Pepitome: Evaluating Improved Spectral Library Search for Identification Complementarity and Quality Assessment. *J. Proteome Res.* **2012**, *11*, 1686–1695. [[CrossRef](#)] [[PubMed](#)]
123. Van Steendam, K.; De Wulf, O.; Dhaenens, M.; Deforce, D. Species identification from hair by means of spectral library searches. *Int. J. Legal Med.* **2014**, *128*, 873–878. [[CrossRef](#)]
124. Muth, T.; Hartkopf, F.; Vaudel, M.; Renard, B.Y. A potential golden age to come—Current tools, recent use cases, and future avenues for de novo sequencing in proteomics. *Proteomics* **2018**, *18*, 1700150. [[CrossRef](#)]

125. Taylor, J.A.; Johnson, R.S. Sequence database searches via de novo peptide sequencing by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1067–1075. [\[CrossRef\]](#)
126. Dančák, V.; Addona, T.A.; Clauser, K.R.; Vath, J.E.; Pevzner, P.A. De novo peptide sequencing via tandem mass spectrometry. *J. Comput. Biol.* **1999**, *6*, 327–342. [\[CrossRef\]](#) [\[PubMed\]](#)
127. Ma, B.; Zhang, K.; Hendrie, C.; Liang, C.; Li, M.; Doherty-Kirby, A.; Lajoie, G. PEAKS: Powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2337–2342. [\[CrossRef\]](#)
128. Frank, A.; Pevzner, P. PepNovo: De Novo peptide sequencing via probabilistic network modeling. *Anal. Chem.* **2005**, *77*, 964–973. [\[CrossRef\]](#)
129. Fischer, B.; Roth, V.; Roos, F.; Grossmann, J.; Baginsky, S.; Widmayer, P.; Gruissem, W.; Buhmann, J.M. NovoHMM: A hidden markov model for de novo peptide sequencing. *Anal. Chem.* **2005**, *77*, 7265–7273. [\[CrossRef\]](#)
130. Tran, N.H.; Zhang, X.; Xin, L.; Shan, B.; Li, M. De novo peptide sequencing by deep learning. *Proc. Nat. Acad. Sci. USA* **2017**, *114*, 8247–8252. [\[CrossRef\]](#)
131. Lee, C.-H.; Kim, M.-S.; Chung, B.M.; Leahy, D.J.; Coulombe, P.A. Structural basis for heteromeric assembly and perinuclear organization of keratin filaments. *Nat. Struct. Mol. Biol.* **2012**, *19*, 707–715. [\[CrossRef\]](#)
132. Bengtsson, C.F.; Olsen, M.E.; Brandt, L.Ø.; Bertelsen, M.F.; Willerslev, E.; Tobin, D.J.; Wilson, A.S.; Gilbert, M.T.P. DNA from keratinous tissue. Part I: Hair and nail. *Ann. Anat. Anat. Anz.* **2012**, *194*, 17–25. [\[CrossRef\]](#)
133. Catlin, L.A.; Chou, R.M.; Goecker, Z.C.; Mullins, L.A.; Silva, D.; Spurbeck, R.R.; Parker, G.J.; Bartling, C.M. Demonstration of a mitochondrial DNA-compatible workflow for genetically variant peptide identification from human hair samples. *Forensic Sci. Int. Genet.* **2019**, *43*, 102148. [\[CrossRef\]](#)
134. Zhang, Z.; Burke, M.C.; Wallace, W.E.; Liang, Y.; Sheetlin, S.L.; Mirokhin, Y.A.; Tchekhovskoi, D.V.; Stein, S.E. Sensitive method for the confident identification of genetically variant peptides in human hair keratin. *J. Forensic Sci.* **2020**, *65*, 406–420. [\[CrossRef\]](#)
135. Kidd, K.K.; Pakstis, A.J.; Speed, W.C.; Grigorenko, E.L.; Kajuna, S.L.B.; Karoma, N.J.; Kungulilo, S.; Kim, J.-J.; Lu, R.-B.; Odunsi, A.; et al. Developing a SNP panel for forensic identification of individuals. *Forensic Sci. Int.* **2006**, *164*, 20–32. [\[CrossRef\]](#)
136. Sheynkman, G.M.; Shortreed, M.R.; Frey, B.L.; Scalf, M.; Smith, L.M. Large-scale mass spectrometric detection of variant peptides resulting from nonsynonymous nucleotide differences. *J. Proteome Res.* **2014**, *13*, 228–240. [\[CrossRef\]](#) [\[PubMed\]](#)
137. Carlson, T.L.; Moini, M.; Eckenrode, B.A.; Allred, B.M.; Donfack, J. Protein extraction from human anagen head hairs 1-millimeter or less in total length. *BioTechniques* **2018**, *64*, 170–176. [\[CrossRef\]](#) [\[PubMed\]](#)
138. Franklin, R.N.; Karim, N.; Goecker, Z.C.; Durbin-Johnson, B.P.; Rice, R.H.; Parker, G.J. Proteomic genotyping: Using mass spectrometry to infer SNP genotypes in pigmented and non-pigmented hair. *Forensic Sci. Int.* **2020**, *310*, 110200. [\[CrossRef\]](#) [\[PubMed\]](#)
139. Parker, G.; Goecker, Z.; Franklin, R.; Durbin-Johnson, B.; Milan, J.; Karim, N.; De Leon, C.; Matzoll, A.; Borja, T.; Rice, B. Proteomic genotyping: Using mass spectrometry to infer SNP genotypes in a forensic context. *Forensic Sci. Int. Genet. Suppl. Ser.* **2019**, *7*, 664–666. [\[CrossRef\]](#)
140. Chu, F.; Mason, K.E.; Anex, D.S.; Jones, A.D.; Hart, B.R. Proteomic characterization of damaged single hairs recovered after an explosion for protein-based human identification. *J. Proteome Res.* **2020**, *19*, 3088–3099. [\[CrossRef\]](#)
141. Plott, T.J.; Karim, N.; Durbin-Johnson, B.P.; Swift, D.P.; Scott Youngquist, R.; Salemi, M.; Phinney, B.S.; Rocke, D.M.; Davis, M.G.; Parker, G.J.; et al. Age-related changes in hair shaft protein profiling and genetically variant peptides. *Forensic Sci. Int. Genet.* **2020**, *47*, 102309. [\[CrossRef\]](#)
142. Clarke, B. Normal bone anatomy and physiology. *Clin. J. Am. Soc. Nephrol.* **2008**, *3*, S131–S139. [\[CrossRef\]](#)
143. Choi, N.-R.; Sándor, G.K.; Kim, Y.-D. Efficacy of collagen-based membranes in alveolar bone augmentation. *Appl. Sci.* **2018**, *8*, 2048. [\[CrossRef\]](#)
144. Buckley, M.; Whitcher Kansa, S.; Howard, S.; Campbell, S.; Thomas-Oates, J.; Collins, M. Distinguishing between archaeological sheep and goat bones using a single collagen peptide. *J. Archaeol. Sci.* **2010**, *37*, 13–20. [\[CrossRef\]](#)
145. Wadsworth, C.; Buckley, M. Proteome degradation in fossils: Investigating the longevity of protein survival in ancient bone. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 605–615. [\[CrossRef\]](#) [\[PubMed\]](#)
146. Ishihama, Y.; Oda, Y.; Tabata, T.; Sato, T.; Nagasu, T.; Rappsilber, J.; Mann, M. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell. Proteom.* **2005**, *4*, 1265–1272. [\[CrossRef\]](#) [\[PubMed\]](#)
147. Mizukami, H.; Hathway, B.; Procopio, N. Aquatic decomposition of mammalian corpses: A forensic proteomic approach. *J. Proteome Res.* **2020**, *19*, 2122–2135. [\[CrossRef\]](#)
148. Johnston, E.; Buckley, M. Relative protein abundances and biological ageing in whole skeletal elements. *J. Proteome Res.* **2020**. [\[CrossRef\]](#)
149. Yang, H.; Zhou, B.; Deng, H.; Prinz, M.; Siegel, D. Body fluid identification by mass spectrometry. *Int. J. Legal Med.* **2013**, *127*, 1065–1077. [\[CrossRef\]](#)
150. Virkler, K.; Lednev, I.K. Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene. *Forensic Sci. Int.* **2009**, *188*, 1–17. [\[CrossRef\]](#)
151. Harbison, S.; Fleming, R. Forensic body fluid identification: State of the art. *Res. Rep. Forensic Med. Sci.* **2016**, *6*, 11–23. [\[CrossRef\]](#)

152. Whiteaker, J.R.; Lin, C.; Kennedy, J.; Hou, L.; Trute, M.; Sokal, I.; Yan, P.; Schoenherr, R.M.; Zhao, L.; Voytovich, U.J.; et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat. Biotechnol.* **2011**, *29*, 625–634. [[CrossRef](#)] [[PubMed](#)]
153. Wild, B.J.; Green, B.N.; Cooper, E.K.; Lalloz, M.R.A.; Erten, S.; Stephens, A.D.; Layton, D.M. Rapid identification of hemoglobin variants by electrospray ionization mass spectrometry. *Blood Cells Mol. Dis.* **2001**, *27*, 691–704. [[CrossRef](#)]
154. Espinoza, E.O.; Lindley, N.C.; Gordon, K.M.; Ekhooff, J.A.; Kirms, M.A. Electrospray ionization mass spectrometric analysis of blood for differentiation of species. *Anal. Biochem.* **1999**, *268*, 252–261. [[CrossRef](#)]
155. Lindahl, M.; Ståhlbom, B.; Tagesson, C. Identification of a new potential airway irritation marker, palate lung nasal epithelial clone protein, in human nasal lavage fluid with two-dimensional electrophoresis and matrix-assisted laser desorption/ionization-time of flight. *Electrophoresis* **2001**, *22*, 1795–1800. [[CrossRef](#)]
156. Sun, W.; Li, F.; Wu, S.; Wang, X.; Zheng, D.; Wang, J.; Gao, Y. Human urine proteome analysis by three separation approaches. *Proteomics* **2005**, *5*, 4994–5001. [[CrossRef](#)] [[PubMed](#)]
157. Doykov, I.D.; Heywood, W.E.; Nikolaenko, V.; Śpiewak, J.; Hällqvist, J.; Clayton, P.T.; Mills, P.; Warnock, D.G.; Nowak, A.; Mills, K. Rapid, proteomic urine assay for monitoring progressive organ disease in Fabry disease. *J. Med. Genet.* **2020**, *57*, 38–47. [[CrossRef](#)] [[PubMed](#)]
158. Legg, K.M.; Powell, R.; Reisdorph, N.; Reisdorph, R.; Danielson, P.B. Discovery of highly specific protein markers for the identification of biological stains. *Electrophoresis* **2014**, *35*, 3069–3078. [[CrossRef](#)] [[PubMed](#)]
159. Kushner, I.K.; Clair, G.; Purvine, S.O.; Lee, J.-Y.; Adkins, J.N.; Payne, S.H. Individual variability of protein expression in human tissues. *J. Proteome Res.* **2018**, *17*, 3914–3922. [[CrossRef](#)] [[PubMed](#)]
160. Carvalho, P.C.; Fischer, J.S.G.; Chen, E.I.; Yates, J.R.; Barbosa, V.C. PatternLab for proteomics: A tool for differential shotgun proteomics. *BMC Bioinform.* **2008**, *9*, 316. [[CrossRef](#)] [[PubMed](#)]
161. Kuhring, M.; Renard, B.Y. iPiG: Integrating peptide spectrum matches into genome browser visualizations. *PLoS ONE* **2012**, *7*, e50246. [[CrossRef](#)]
162. Li, C.; Li, K.; Li, K.; Xie, X.; Lin, F. SWPepNovo: An efficient de novo peptide sequencing tool for large-scale MS/MS spectra analysis. *Int. J. Biol. Sci.* **2019**, *15*, 1787. [[CrossRef](#)] [[PubMed](#)]
163. Manguy, J.; Jehl, P.; Dillon, E.T.; Davey, N.E.; Shields, D.C.; Holton, T.A. Peptigram: A web-based application for peptidomics data visualization. *J. Proteome Res.* **2017**, *16*, 712–719. [[CrossRef](#)]
164. Vitorino, R. Digging deep into peptidomics applied to body fluids. *Proteomics* **2018**, *18*, 1700401. [[CrossRef](#)]
165. Brown, C.O.; Robbins, B.L.; McKiernan, H.E.; Danielson, P.B.; Legg, K.M. Direct seminal fluid identification by protease-free high-resolution mass spectrometry. *J. Forensic Sci.* **2020**. [[CrossRef](#)] [[PubMed](#)]
166. Timms, M.; Steel, R.; Vine, J. Identification of recombinant human EPO variants in greyhound plasma and urine by ELISA, LC-MS/MS and western blotting: A comparative study. *Drug Test. Anal.* **2016**, *8*, 164–176. [[CrossRef](#)] [[PubMed](#)]
167. Skiba, M.; Fatmi, S.; Elkasri, N.; Karrouit, Y.; Lahiani-Skiba, M. Development, validation and method stability study of a LC-MS method to quantify leuprolide (Hormone analog) in human plasma. *J. Chromatogr. B* **2020**, *1160*, 122345. [[CrossRef](#)]
168. Botrè, F. Mass spectrometry and illicit drug testing: Analytical challenges of the anti-doping laboratories. *Expert Rev. Proteomics* **2008**, *5*, 535–539. [[CrossRef](#)]
169. Pittner, S.; Monticelli, F.C.; Pfisterer, A.; Zissler, A.; Sängler, A.M.; Stoiber, W.; Steinbacher, P. Postmortem degradation of skeletal muscle proteins: A novel approach to determine the time since death. *Int. J. Legal Med.* **2016**, *130*, 421–431. [[CrossRef](#)]
170. Pittner, S.; Gotsmy, W.; Zissler, A.; Ehrenfellner, B.; Baumgartner, D.; Schröfer, A.; Steinbacher, P.; Monticelli, F. Intra- and intermuscular variations of postmortem protein degradation for PMI estimation. *Int. J. Legal Med.* **2020**, *134*, 1775–1782. [[CrossRef](#)]
171. Huculak, M.A.; Rogers, T.L. Reconstructing the sequence of events surrounding body disposition based on color staining of bone. *J. Forensic Sci.* **2009**, *54*, 979–984. [[CrossRef](#)]
172. Wescott, D.J. Recent advances in forensic anthropology: Decomposition research. *Forensic Sci. Res.* **2018**, *3*, 278–293. [[CrossRef](#)] [[PubMed](#)]
173. Sarabia, J.; Pérez-Martínez, C.; Hernández del Rincón, J.P.; Luna, A. Study of chemiluminescence measured by luminometry and its application in the estimation of postmortem interval of bone remains. *Legal Med.* **2018**, *33*, 32–35. [[CrossRef](#)]
174. Pérez-Martínez, C.; Pérez-Cárceles, M.D.; Legaz, I.; Prieto-Bonete, G.; Luna, A. Quantification of nitrogenous bases, DNA and Collagen type I for the estimation of the postmortem interval in bone remains. *Forensic Sci. Int.* **2017**, *281*, 106–112. [[CrossRef](#)]
175. Ferreira, P.G.; Muñoz-Aguirre, M.; Reverter, F.; Sá Godinho, C.P.; Sousa, A.; Amadoz, A.; Sodaie, R.; Hidalgo, M.R.; Pervouchine, D.; Carbonell-Caballero, J.; et al. The effects of death and post-mortem cold ischemia on human tissue transcriptomes. *Nat. Commun.* **2018**, *9*, 490. [[CrossRef](#)] [[PubMed](#)]
176. Jarman, K.H.; Heller, N.C.; Jenson, S.C.; Hutchison, J.R.; Kaiser, B.L.D.; Payne, S.H.; Wunschel, D.S.; Merkley, E.D. Proteomics Goes to Court: A statistical foundation for forensic toxin/organism identification using bottom-up proteomics. *J. Proteome Res.* **2018**, *17*, 3075–3085. [[CrossRef](#)] [[PubMed](#)]
177. Abboud, A. Daubert v. merrell dow pharmaceuticals, inc.(1993). *Embryo Project Encycl.* 2017. Available online: <https://embryo.asu.edu/pages/daubert-v-merrell-dow-pharmaceuticals-inc-1993> (accessed on 20 February 2021).
178. Jarman, K.H.; Merkley, E.D. The Statistical Defensibility of Forensic Proteomics. In *Applications in Forensic Proteomics: Protein Identification and Profiling*; American Chemical Society: Washington, DC, USA, 2019; Volume 1339, pp. 203–228.