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Multiple Reaction Monitoring Tandem Mass Spectrometry Approach for the Identification of Biological Fluids at Crime Scene Investigations

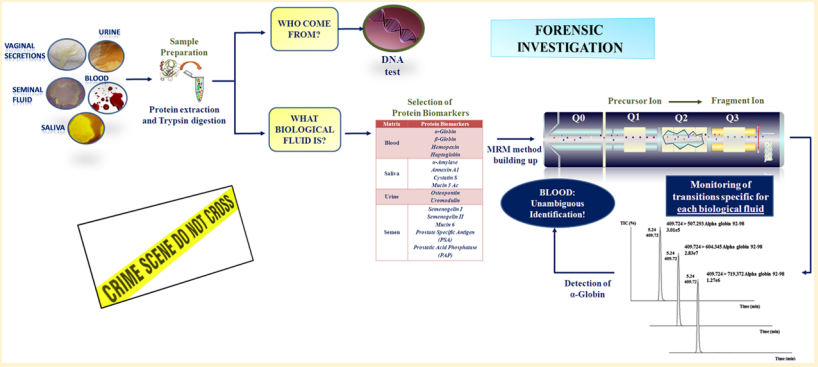
Anna Illiano,*[,†] Valentina Arpino,[†] Gabriella Pinto,[†] Andrea Berti,[‡] Vincenzo Verdoliva,[‡] Giuseppe Peluso,[§] Piero Pucci,[†] and Angela Amoresano[†]

[†]Dipartimento di Scienze Chimiche, Università di Napoli Federico II, Napoli 80126, Italy

[‡]Carabinieri, Reparto Investigazioni Scientifiche (R.I.S.) di Roma, Viale di Tor di Quinto n. 151, Roma 00191, Italy

[§]Carabinieri, Sezione Investigazioni Scientifiche (S.I.S.) di Napoli, Corso Vittorio Emanuele n. 728, Napoli 80122, Italy

Supporting Information



ABSTRACT: Knowledge of the nature of biofluids at a crime scene is just as important as DNA test to link the nature of the biofluid, the criminal act, and the dynamics of the crime. Identification of methods currently used for each biological fluid (blood, semen, saliva, urine) suffer from several limitations including instability of assayed biomolecules, and low selectivity and specificity; as an example of the latter issue, it is not possible to discriminate between alpha-amylase 1 (present in saliva) and alpha-amylase 2 (present in semen and vaginal secretion. In this context, the aim of the work has been to provide a predictive protein signature characteristic of each biofluid by the recognition of specific peptides unique for each protein in a single analysis. A panel of four protein biomarkers for blood, four for saliva, five for semen, and two for urine has been monitored has been monitored by using a single multiple reaction monitoring (MRM)-based method targeting concomitantly 46 different peptides. Then, The optimized method allows four biological matrices to be identified when present on their own or in 50:50 mixture with another biofluid. Finally, a valid strategy combining both DNA analysis and liquid chromatographic-tandem mass spectrometric multiple reaction monitoring (LC-MS-MRM) identification of biofluids on the same sample has been demonstrated to be particularly effective in forensic investigation of real trace evidence collected at a crime scene.

In the last three decades the link between forensic investigations and scientific disciplines, such as Chemistry and Biology, has been enormously strengthened by the application of new tools for a detailed characterization of the crime scene starting from trace evidence. In particular, DNA testing is fundamental to identify the individuals involved in a crime often leading the investigators to define a unique genetic profile.[1]

However, the detection and identification of the type and origin of biofluids at a crime scene are just as important as DNA test to link the nature of the biofluid, the criminal act and the dynamics of the crime. For example, blood stains can indicate some form of physical altercation, whereas detection of semen or vaginal fluid can indicate the involvement of some form of sexual encounter or assault. Moreover, identification of the specific fluid or tissue from which the DNA sample was recovered is fundamental in correctly reconstructing the criminal event and the effective role of the donor.

The most common biofluids found at crime scenes are blood, semen, and saliva, but others such as vaginal fluid, urine, and sweat can also be found. Each of these fluids has one or more presumptive tests that are initially used to give some indication as to the identity of the substance; blood is quickly detected by means of luminol or benzidine,[2] whereas traces of semen, saliva, sweat, urine, and other biological fluids are detected by ultraviolet light or by other light sources at specific wavelengths.[3] Confirmatory tests, based on biochemical, spectroscopic, and

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microscopy methods, and immunochemical techniques, are necessary to give a legal value to the trace evidence.[4−9]

However, these identification methods suffer from several limitations including instability of assayed biomolecules and low selectivity and specificity. As an example of the latter issue, it is not possible to discriminate between α-amylase 1 (present in saliva) and 2 (present in semen and vaginal secretion).[10] Moreover, biochemical assays are specific only for one biological matrix and several cascade tests might then be needed before the biological nature of a certain stain is uncovered. This is even more challenging when the sample is a mixture of different biological matrices thus greatly increasing the complexity, the cost and the time of the analytical procedures. Finally, some confirmatory tests very often cause sample loss and they are not compatible with downstream individual identification by DNA analysis.

For all these issues, forensic science is looking for a universal confirmatory test for the analysis of unknown stains which will be able to unambiguously identify the type and nature of any biofluids that might be present at a crime scene. The method should be applicable to mixtures and, more importantly, should preserve the samples for subsequent DNA analysis. Since biofluids have evolved to perform different functions, they contain different proteins, or different combinations of proteins, providing each biological matrix with a unique protein signature that can be used to distinguish among the various biofluids.[11−13] Recently, untargeted proteomics have been introduced for the determination of biological matrices in forensic science based on the identification of the most prominent proteins present in biofluids.[11,14] However, these methods can give poor or uncertain results when high amounts of non specific proteins occur in the samples, i.e. when low amounts of a particular fluid are present in combination with high amounts of other matrices or when the sample is contaminated by other tissues.

This Article reports a universal method alternative and complementary to "traditional" tests currently used in forensic investigation to identify an unknown stain of biofluids occurring at a crime scene. The method is based on a targeted proteomic approach that makes use of tandem mass spectrometry in multiple reaction monitoring (MRM) mode to selectively monitor a number of specific peptides belonging to proteins biomarkers of individual biological fluids. First, the most prominent and highly specific protein biomarkers for each biological matrix were identified by both conventional LC-MS/MS analyses and comparison with literature data. Specific peptide markers of each protein were then selected on the basis of their mass spectrometric behavior together with their specific precursor ion-product ion transitions as defined by their unique amino acid sequence. Then, a single MRM method was devised to detect the occurrence of the target peptides within the sample leading to the unambiguous discrimination among the different biological matrices in a single analysis because of its high sensitivity, selectivity and accuracy. The optimized method was tested and validated on specimens consisting of four biological matrices (blood, saliva, semen, and urine), or mixtures of matrices, spotted on different substrates like cloth, wood, plastic, plaster, and paper. Finally, a combined strategy allowing both DNA analysis and biofluids identification on the same sample was developed and demonstrated to be effective in forensic investigation by the analysis of real specimens collected at a crime scene.

MATERIALS AND METHODS

Control biofluids (blood, saliva, urine, semen) were provided by a private chemical laboratory. Guanidine, Tris(hydroxymethyl) aminomethane hydrochloride, dithiothreitol (DTT), ethylendiaminetetraacetate (EDTA), trypsin, iodoacetamide (IAM), ammonium bicarbonate (AMBIC), trichloroacetic acid (TCA) were purchased from Sigma-Aldrich. Bovine serum albumin (BSA), formic acid (HCOOH), methanol, chloroform, and acetonitrile (ACN) are from J.T. Baker. Bradford staining solution was from Bio-Rad. Pipette tips C18 (zip tip) and centrifugal filter units were purchased from Merck Millipore.

In-Solution Digestion of Proteins from Biological Matrices. Different protocols were performed to improve the number of identified proteins and thus to provide the higher sequence coverage for blood, urine, semen, and saliva. A protocol of protein precipitation by using chloroform/methanol/water[15] was carried out for each biofluids. After precipitation, protein concentration was determined by Bradford assay using BSA as standard.[16] Samples were submitted to reduction, alkylation and tryptic digestion. Samples were dissolved in denaturant buffer (urea 6 M, Tris 300 mM pH 8.0, EDTA 10 mM) containing DTT (10-fold molar excess on the Cys residues) at 37 °C for 2 h and then iodoacetamide (IAM) was added to perform carboamidomethylation using an excess of alkylating agent (5-fold molar excess on thiol residues). The mixture was then incubated in the dark at room temperature for 30 min. The product was purified by chloroform/methanol/water precipitation. Supernatants were removed and the pellets were dried. Digestion of proteins mixture was carried out in AMBIC 10 mM using trypsin at a 50:1 protein/enzyme mass ratio. The samples were incubated at 37 °C for 16 h and after acidification (10% HCOOH) they were dried. To eliminate any impurities the samples were suspended in 200 μL of AMBIC 100 mM, filtrated by centrifugal filter units (0.22 μm), and dried in a speed-vacuum concentrator. Finally, samples were suspended in 20 μL of HCOOH 1% and purified by reverse phase chromatography using ZipTip C18 cartridges (Millipore). Samples were evaporated and suspended in 10 μL of HCOOH 0.1% and analyzed by nanoLC-MS/MS and then by LC-MRM/MS as described below.

To improve the number of identified proteins from blood by LC-MS/MS, a step of depletion of abundant proteins was carried out using ProteoPrepR 20 Plasma Immunodepletion KitProteoprep 20 Plasma Immunodepletion Kit (Sigma-Aldrich, Milan, Italy) able to deplete 20 highly abundant proteins from human plasma or serum. Dried extracted proteins were subjected to reduction, carboamidomethylation, chloroform/methanol/water precipitation protocol and tryptic hydrolysis as described above.

Protein precipitation from urine was also performed using cold acetone followed by the same steps of tryptic digestion as described above. Cold acetone at −20 °C (400 μL) was added to urine (100 μL) and incubated at −20 °C for 2 h. After centrifugation at 12 000 rpm for 15 min the pellet was collected and dried under vacuum.

Protein precipitation from semen was also carried out using TCA. Semen was centrifuged (13 000 rpm, 30 min) to remove cellular material, and the proteins were precipitated (4 °C, 60 min) using an equal volume of cold TCA at final concentration of 10%. Following further centrifugation (10 000 rpm, 30 min), the supernatant was removed and the protein pellet washed with cold acetone (three times) to remove residual TCA.

Therefore, a unique precipitation/sample preparation procedure based on chloroform/methanol/water was adopted for all the samples because the origin or matrix of casework samples.

Test Specimens. Twenty test forensic samples were prepared by drying different biological fluids (100 μL) or mixture of them on various substrates: cloth, wood, plastic,

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plaster and paper. Cloths were put directly in a plastic tube while the samples on the other substrates were recovered by a cotton-swab (Agilent Technologies, Palo Alto, CA). Samples were collected in plastic tubes and 2 mL of AMBIC 50 mM were added for 16 h under gentle agitation. Samples were then sonicated for 20 min and submitted to chloroform/methanol/water precipitation. After precipitation, reduction, alkylation and tryptic digestion were performed as previously described. The resulting peptide mixtures were analyzed by LC-MRM/MS as described below.

Analysis of Real Samples. Real samples consisting of traces of unknown biological fluids on cloths, paper and stubs collected at a crime scene (samples 1, 2, and 3) were provided by RIS Laboratory in Rome. Samples were treated as described above (see Test Specimens section) and submitted to tryptic digestion. Then, gDNA was extracted from the samples using the robotic platform Qiagen Biorobot EZ1 Advanced XL using the Qiagen EZ1 DNA Investigator kit.[17] The DNA was then washed and eluted in water. DNA degradation and quantification level was evaluated by multiplex RT-PCR using the Quantifiler Trio DNA Quantification Kit (Applied Biosystems, CA, USA)[18] on a 7500 Real-Time PCR System (Applied Biosystems). Results showed a total DNA amount of 0.081 ng/μL for sample 1, 0.71 ng/μL for sample 2, and 1.05 ng/μL for sample 3. Samples were also tested for possible DNA degradation showing a good quality with a degradation index of about 1.0 for all samples.

DNA profiling was obtained by multiple amplification of 17 polimorphic regions comprising the D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, TH01, D16S539, D2S1338, D19S433, ACTBP2 (SE33), D1S1656, D2S441, D10S1248, D12S391, D22S1045, and Amelogenin STR loci using the Investigator ES Splex SE Plus Kit Qiagen (Qiagen, Hilden, Germany).[18] Amplified DNA was analyzed by automated DNA sequencing on an Applied Biosystems 3500xL Genetic Analyzer.[19−21] Data were collected and elaborated using the 3500 Series Data Collection Software v. 2.0and the Gene Mapper ID-X Software v.1.4 (Life Technologies, Carlsbad, CA, USA).

LC-MS/MS Analysis. Peptide mixture were analyzed by LC-MS/MS on a 6520 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture (1 μL) was concentrated and desalted at flow rate of 4 μL/min in a 40 nL enrichment column (Agilent Technologies chip) with 0.1% HCOOH as eluent. The sample was then fractionated on a C18 reverse phase capillary column (75 μm*43 mm in the Agilent Technologies chip) at flow rate of 400 nL/min, with a linear gradient of eluent B (0.1% HCOOH in 95% acetonitrile) in A (0.1% HCOOH in 2% acetonitrile) from 5% to 80% in 50 min. Peptides analysis was performed using data-dependent acquisition of one MS scan (mass range m/z 300−2400) followed by MS/MS scan of the five most abundant ions in each MS scan. MS/MS spectra were measured automatically when the MS signal was greater than the threshold of 50000 counts. Doubly- and triply- charged ions were preferably isolated and fragmented over singly charged ions. Data were acquired through Mass Hunter software (Agilent Technologies). The acquired data, containing MS and MS/MS spectra, were transformed in .mgf format and used for protein identification with a licensed version of Mascot Software (www.matrixscience.com).

Mascot search parameters included: NCBInr as database; trypsin as enzyme, allowed number of missed cleavage 3; *Homo-Sapiens* as taxonomy; carbamidomethyl, C as fixed modifications; oxidation of methionine (oxidation (M));, *Gln pyro-Glu* (N-term Q) as variable modifications; 10 ppm MS tolerance, 0.6 Da MS/MS tolerance and peptide charge, from +2 to +3.

MRM Targeted Proteomic Approach. To build up a targeted MRM method, Skyline software (3.7, 64 bit version MacCoss Lab Software, University of Washington, USA) was used for the in silico selection of peptides with unique sequence for each selected protein. For each peptide, m/z precursor ion, m/z product ions and relative collision energy were provided by Skyline. Peptide mixture was analyzed by LC-MS/MS analysis using a Xevo TQ-S (Waters) equipped with an IonKey UPLC Microflow Source coupled to an UPLC Acquity System (Waters). For each run, 1 μL peptide mixture was injected and separated on a TS3 1.0 mm × 150 mm analytical RP column (Waters, Milford, MA, USA) at 45 °C with flow rate of 3 μL/min using 0.1% HCOOH in water (LC-MS grade) as eluent A and 0.1% HCOOH in ACN as eluent B. Peptides were eluted (starting 1 min after injection) with a linear gradient of eluent B in A from 7% to 95% in 55 min. The column was re-equilibrated at initial conditions for 4 min. The MRM mass spectrometric analyses were performed in positive ion mode using a MRM detection window of 0.5−1.6 min per peptide; the duty cycle was set to automatic and dwell times were minimal 5 ms. Cone voltage was set to 35 V.

RESULTS

Selection of Protein Biomarkers of Biological Matrices. Four biological matrices, blood, saliva, urine, and semen, representative of biofluids recovered at crime scenes, were analyzed to identify specific protein biomarkers candidates for each matrix. Different procedures for sample preparation were investigated (Material and Methods section). As an example, results obtained for blood with the only chloroform/methanol/water precipitation followed by tryptic hydrolysis, are shown in Table 1 where

Table 1. List of the Identified Proteins Following the Chloroform/Methanol/Water Precipitation Protocol Applied to Human Blood Sample

GI number	identification	MascotScore	number of peptides	sequence coverage (%)
gil3212456	chain A, crystal structure of human serum albumin	1186	35	24
gil 90108664	chain A, crystal structure of lipid-free human apolipoprotein A-I	602	18	22
gil2765421	immunoglobulin kappa heavy chain	192	4	34
gil28637	alpha-1 antitrypsin	188	5	5
gil386789	hemopexin precursor	178	7	10
gil28810	β-2-glycoprotein apolipoprotein H	160	6	1
gil4557871	serotransferrin precursor	146	6	5
gil38026	Zn-α2-glycoprotein	75	2	5
gil112910	α-2-HS-glycoprotein	71	2	4

GI number, the name of identified proteins, the number of peptides and the corresponding sequence coverage are reported.

The unsatisfactory number of identified proteins in the original assay severely limited the choice of specific protein biomarkers for blood candidates for the MRM method because the dominant abundance of unselective blood proteins. Thus, a different sample preparation procedure was then explored by a depletion step from the most abundant proteins to improve protein identification (Table 2). A significantly higher number of

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Table 2. List of the Identified Proteins Following the Depletion Step Previous to the Chloroform/Methanol/Water Precipitation Protocol[a]

GI number	identification	MascotScore	number of peptides	sequence coverage (%)
gil1431650	chain β of hemoglobin	156	6	20
gil229751	chain α of hemoglobin	138	4	15
gil3212456	chain A, crystal structure of human serum albumin	1995	48	34
gil 194383506	serum transferrin	1012	34	29
gil90108664	chain A, crystal structure of lipid-free human apolipoprotein A-I	562	22	27
gil177870	α-2-macroglobulin	485	17	7
gil13787109	α-1-antitrypsin	337	12	14

[a]The GI number, the number of peptides, the corresponding Mascot score, and sequence coverage are reported for human blood sample.

peptides were identified for each protein leading to a much larger sequence coverage.

Improved results were also obtained in saliva, semen, and urine analysis by using the chloroform/methanol/water precipitation protocol. Table S-1 reports the lists of the different proteins identified for the other matrices, including the GI number, the number of peptides and the Mascot score.

Proteins identified for each biofluids by LC-MS/MS were then compared with literature data[11] to eventually select the most specific protein biomarkers for each biological matrix. Following this procedure, proteins listed in Table S-2 were chosen as representative for each biological fluid.

Development of MRM Methods Specific for Each Biological Matrix and a Single MRM Method for All the Matrices. A number of unique peptides belonging to the target proteins characteristic of individual biological matrix were selected by in silico analysis using the Skyline software that provided the predicted best transitions and collision energy to generate maximal fragment intensities. The in silico data of the selected peptides were then compared with the experimental fragmentation spectra obtained by the LC-MS/MS analyses. Peptides defined by Skyline analysis and showing the best signal-to-noise ratio in the experimental fragmentation spectra were selected for developing MRM methods for each biological fluid. These methods contained all the precursor ion−product ions transitions and collision energy associated with the selected peptides from the defined target proteins constituting the protein signature of each individual biofluids in Table S-2.

As an example, two peptides from α-globin, three from β-globin and hemopexin, five from haptoglobin, and six from α-2-macroglobulinfor a total of 25 peptides and 106 transitions were selected to monitor the presence of blood and to build the MRM method for this biological matrix.

The total ion current chromatogram (A) and the MRM TIC analysis of the three peptides 132−143, 82−94, and 30−39 from β-globin (B) are shown in Figure 1. Panel C displays the MRM TIC chromatogram of the three transitions, m/z 409.72 to 507.29, m/z 409.72 to 604.34, and m/z 409.72 to 719.37, used to monitor the α-globin (92−98) peptide. The different transitions perfectly coeluted at a retention time of 5.24 min thus indicating that they belong to the same precursor ion.

Similar MRM methods were developed for the other biological matrices (Table S-2). For urine, as an example, Figure S-1 shows the total ion current chromatogram (A) and the MRM TIC analysis of the two peptides (507−605) and (204−211) from uromodulin (B). Panel C displays the TIC chromatogram of the three transitions for the uromodulin (204−211) peptide. The different transitions perfectly coeluted at a retention time of 2.99 min thus indicating that they all originated from the same precursor ion.

Some of the MRM TIC chromatograms of the selected peptides for the specific detection of proteins from saliva and semen were reported in Supporting Information (Figures S-2 and S-3).

Once the optimized MRM methods were developed for each individual biological fluid, the next step involved the development of a single MRM method able to detect all the biological fluids in a single analysis. For each selected protein biomarker, all the selected peptide sequences, m/z precursor ion, m/z product ions and the optimized collision energy were tabulated in Table S-4.

As the mass spectrometer was able to handle a very large number of transitions per run, the single MRM method was built up by using a total of 46 peptides and 212 transitions.

Analysis of Test Specimens. For all the test specimen samples, the MRM chromatograms solely showed the mass transitions associated with the target peptides belonging to the specific proteins constituting the unique signature of the defined matrix. No transitions related to peptides from proteins characteristic of other biological fluids were detected for all the analyzed samples. As an example, Figure 2A shows the MRM TIC chromatogram of the three transitions, m/z 637.86 to 687.35, m/z 637.86 to 850.42, m/z 637.86 to 949.49, m/z 637.86 to 1048.56, and m/z 637.86 to 1161.64 used to monitor the β-globin (30−39) peptide form a blood sample spotted on plaster. Panel B showed the corresponding MRM chromatogram for the (1075−1086) peptide from mucin-6, a protein specific of semen, illustrating the absence of any nonspecific transition of interfering proteins. This result clearly demonstrates that the specific signature of blood could be easily monitored whereas no interference from cross transitions of peptides belonging to other biological matrices was detected.

Similar results were obtained on all the other spotted samples and are summarized in Table 3. Each biological matrix could be unambiguously identified by the developed MRM method even on different substrates. No cross transitions were observed whatsoever with the exception of urine where peptides from PSA and PAP proteins, a specific signature of semen, was also detected. However, the presence of seminal proteins in urine is somehow expected and provides further information on the origin of the sample that was obviously from a male donor.

To test the MRM optimized method capability to unambiguously define the occurrence of individual biofluids when occurring in a 50:50 ratio mixture, blood−semen, blood−urine, and semen−urine were spotted onto different substrates. The negative controls, for a total of 5 sample, consisted of the exclusive use of substrates: cloth, wood, plastic, plaster, and paper. As shown in Table S-5, each individual component of the binary mixture was unambiguously identified by the specific transitions of the unique peptide signature; whenever urine was present, specific peptides from semen proteins were also detected.

Analysis of Real Crime Scene Samples. The developed procedure was then applied to real samples collected at a crime scene by the RIS Laboratory in Rome. Samples were prepared according to the procedure described above and following enzymatic hydrolysis, DNA was extracted from each sample according to the normal procedure performed at the RIS

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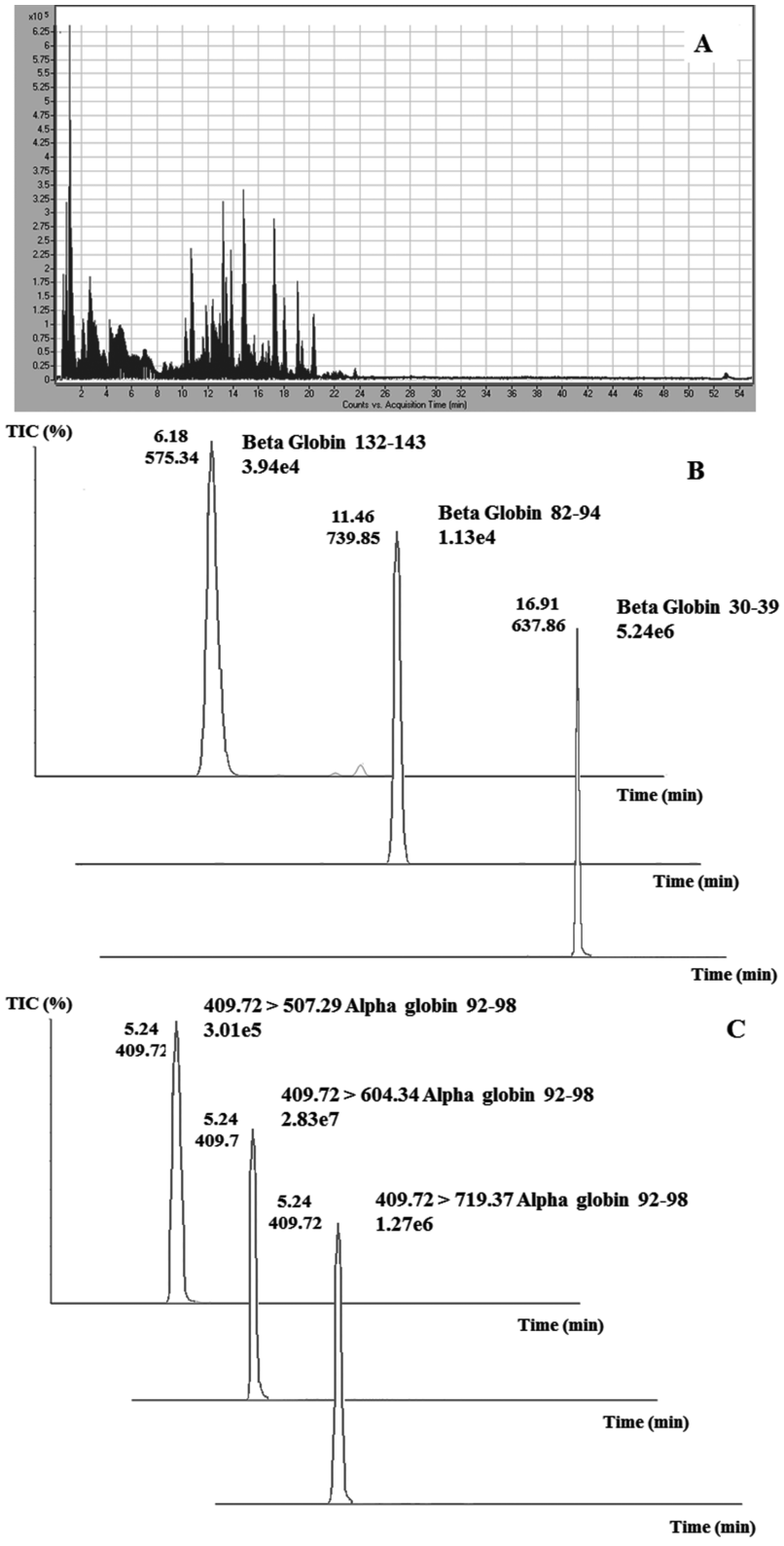

Figure 1. Identification of all the protein of blood fluid by LC-MS/MS analysis and MRM/MS data of the defined target proteins constituting the protein signature of blood. Panel A: Total ion current (TIC) as a function of time (min.). LC-MS-MS analysis allowed the identification of protein content on blood tryptic peptide mixture. Panel B: BPI Chromatograms for β-globin 132−143, 82−94, 30−39 peptides eluted at 6.18, 11.46, and 16.91 min, respectively. Panel C: MRM TIC Chromatograms extracted for α-globin (92−98) peptide. The monitored transitions for this peptide: m/z 409.72 to 719.37, m/z 409.72 to 604.34, and m/z 409.72 to 507.29 are perfectly coeluted at 5.24 min as an unambiguous identification of blood detection.

Laboratory. DNA extracts were quantified, the degradation level was evaluated and the DNA profiling obtained as described in Materials and Methods section. The resulting genotypic profile of sample 1 is shown in Figure S-4 and was identified as a male genotype profile called "Profile A". The quality of the data exceeded the minimum quality requirements established by

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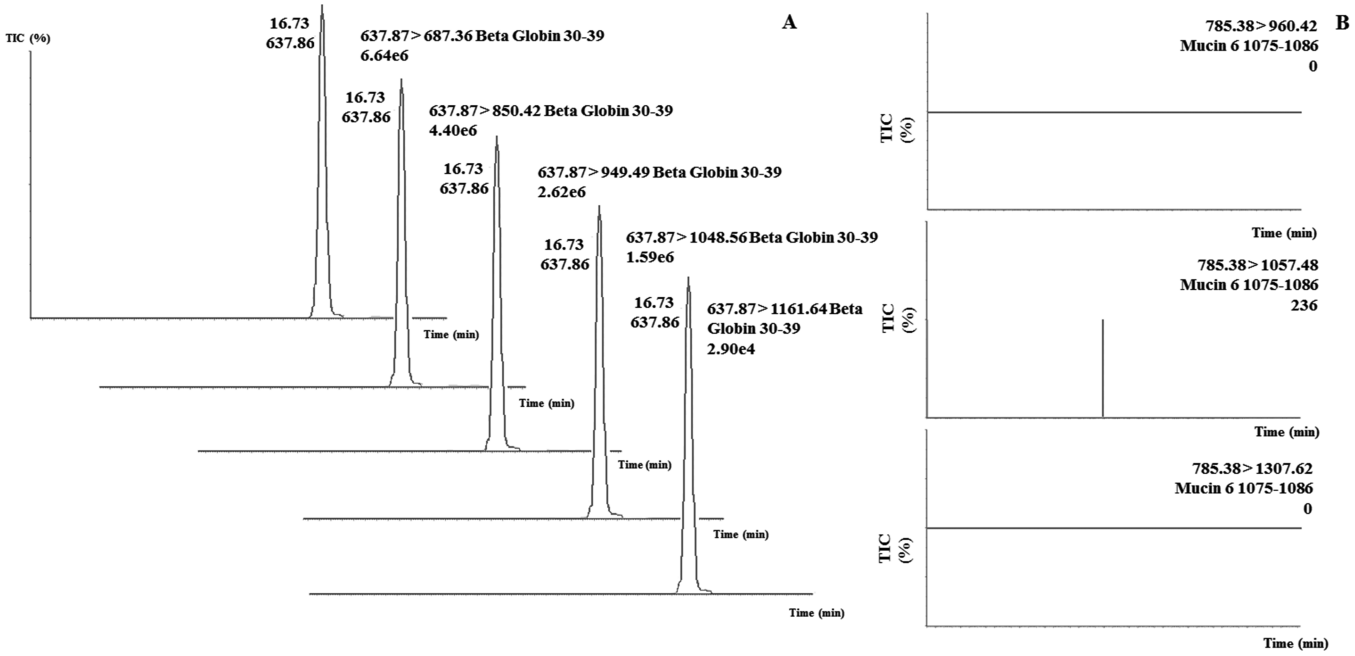

Figure 2. MRM/MS analysis of test specimens: blood spotted on plaster. Panel A: MRM TIC Chromatograms extracted for β-globin (30−39) peptide. The best five monitored transitions m/z 637.87 to 687.36, m/z 637.87 to 850.42, m/z 637.87 to 949.49, m/z 637.87 to 1048.56, and m/z 637.87 to 1161.64 are reported in figure eluted at 16.73 min. Panel B: MRM TIC chromatogram extracted for (1075−1086) peptide from mucin-6 a protein specific of semen, illustrating the absence of any nonspecific transition (m/z 785.38 to 960.42, m/z 785.38 to 1057.48, m/z 785.38 to 1307.62).

internal validation procedures for this Lab for comparative procedures to be used for personal identification purposes.

Following DNA analysis, the remainder of the samples containing the mixture of tryptic peptides was submitted to the developed MRM analytical procedure. Figure 3 shows the TIC analysis of the three peptides 30−39, 82−94, and 132−143 monitored for the human β-globin and two peptides 92−98 and 127−138 monitored for the human α-globin (A). Panel B displays the MRM TIC chromatogram corresponding to four transitions, m/z 637.87 to 687.35, m/z 637.87 to 850.42, m/z 637.87 to 949.48, and m/z 637.87 to 1048.55, used to monitor the β-globin (30−39) peptide. As shown in Table 4, the MRM analysis was able to unambiguously define individual protein component occurring in the different samples by their unique peptide signature even after DNA extraction and analysis. Therefore, this combined procedure led to both DNA profiling analysis and the unambiguous identification of blood indicating the biological fluid the DNA was collected from.

DISCUSSION

Knowledge of the nature of biofluid discovered at a crime scene can influence the outcome of a case. Identification of a suspect's DNA on a victim is quite different if it comes from saliva, suggesting intimate contact, wither consensual or forced, or from blood or seminal fluid, indicating a physical struggle or a sexual assault. However, this is not always an easy task, since many biofluid stains are either invisible to the naked eye or similar in appearance to other fluids or substances. Even when the identity of a stain may seem obvious to a forensic investigator, absolute confirmation is necessary to give a legal value to the trace evidence to either prove or disprove a fact in a lawsuit. This is especially important with the possible occurrence of mixtures as single stain could contain multiple biofluids.

Most biochemical and immunologic tests which have been used for presumptive or confirmatory methods suffer from several limitations leading to sample destruction resulting in sample loss for subsequent DNA analysis or to incompatibility with downstream individual identification assays, that is, DNA profiling. Moreover, most of the current methods are designed to detect a single biofluid forcing the investigators to decide which test to perform in the presence of a limited amount of sample.

Contrary to DNA, proteins were rarely considered and scarcely used as sources of useful biological traces in crime scene investigations. Proteins tend to be less stable than DNA and are easily degraded making their identification by immunological methods unfeasible. Moreover, the amount of available samples cannot be amplified by "PCR-like" procedures thus requiring the use of analytical techniques with extremely high sensitivity.[22]

More recently, proteomics strategies have been applied to the identification of biofluids that overcome most of the previous difficulties. In proteomic approaches, proteins are identified by their peptide fragments making the degradation no longer a problem. Moreover, contrary to DNA, proteins are tissue-specific providing a unique signature to identify biological tissues and fluids. Finally, the tiny amount of trace evidence usually recovered at a crime scene is matched by the extraordinary sensitivity of modern mass spectrometers. Untargeted proteomic approaches demonstrated to be effective in the definition of biofluids in traces from crime scenes by identification of specific proteins belonging to individual biological sample.[5] However, severe limitations in sensitivity, in the analysis of mixed traces and, more important, in preserving the samples for subsequent DNA analysis still exist. A recent study has greatly overcome this drawback by MALD IMS imaging directly on a blood fingermark leaving the ridge detail completely preserved.[10] Despite the fact that the proposed method is as destructive as the other approaches, the great compatibility between DNA extraction and MS strategy protocols allows to limit the destruction of the entire trace evidence. Indeed, a combined strategy to provide both DNA analysis profiling by RIS laboratory and identification of the

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Table 3. List of Proteins Detected in Each Test Specimen Prepared by Drying Different Biological Fluids on Various Substrates: Cloth, Wood, Plastic, Plaster, and Paper[a]

		blood				urine		saliva				semen				
matrixes	substrates	αhemoglobin	βhemoglobin	hemopexin	haptoglobin	uromodulin	osteopontin	α amylase	Muc 5 Ac	annexin A1	custatin S	prostate specificantigen	prostatic acid phosphatase	Muc 6	semenogelin I	semenogelin II
blood	cloth	X	X	X	X											
	wood	X	X	X	X											
	plastic	X	X	X	X											
	plaster	X	X	X	X											
	paper	X	X	X	X											
urine	wood					X	X					X	X			
	plastic					X	X					X	X			
saliva	cloth							X	X	X	X					
	wood							X	X	X	X					
	plastic							X	X	X	X					
	plaster							X	X	X	X					
semen	cloth											X	X	X	X	
	wood											X	X	X		
	plastic											X	X	X	X	

[a]Matrix spotted on substrates. X is reported when the unique peptides belonging to the target proteins characteristic of individual biological matrix were identified by MRM analysis.

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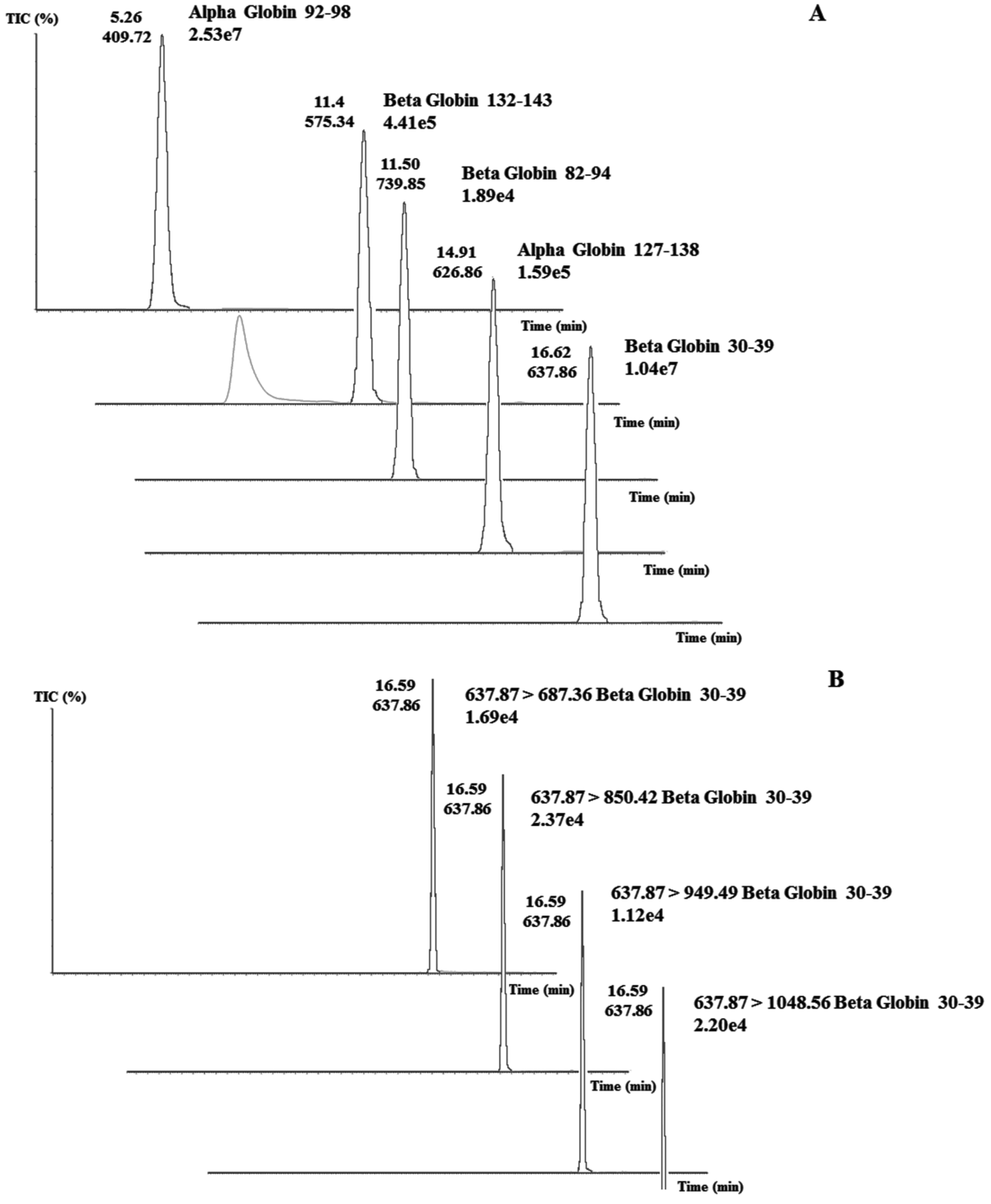

Figure 3. MRM/MS analysis of real crime scene samples after DNA extraction. Panel A: BPI Chromatograms for α-globin 92−98 and 127−138 peptides eluted at 5.26 and 14.91 min respectively and BPI Chromatograms for β-globin 132−143, 82−94, AND 30−39 peptides eluted at 11.40, 11.50, AND 16.62 min, respectively. Panel B: MRM TIC chromatogram corresponding to four transitions, m/z 637.87 to 687.35, m/z 637.87 to 850.42, m/z 637.87 to 949.48, and m/z 637.87 to 1048.55 perfectly coeluted at 16.59 min, used to monitor the β-globin (30−39) peptide.

corresponding biological fluids on the same sample collected at real crime scenes has been developed. Samples were deproteinized by treatment with trypsin instead of the commonly used nonspecific proteases, that is, Pronase, proteinase K, etc. DNA was then extracted from the digested samples and analyzed according to the usual procedure, yielding optimal DNA profiles. Moreover, the proposed *bottom-up* approach makes this method valid also on old traces that have undergone degradation. Another advantage from the current approach is the unambiguous identification of all target proteins allowing us to unequivocally

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Table 4. Identification of the Biomarker Proteins by MRM Analysis in Three Samples Obtained from RIS Laboratories after DNA Extraction and Analysis[a]

		peptide	sample 1	sample 2	sample 3
blood	α Hb	K.VGAHAGEYGAEALER.M [16, 30]	x		
		R.VDPVNFK.L [92, 98]	x		
		K.FLASVSTVLTSK.Y[127, 138]	x		x
	β Hb	R.LLVVYPWTQR.F [30, 39]	x		x
		K.GTFATLSELHCDK.L [82, 94]	x		
		K.VVAGVANALAHK.Y [132, 143]	x		
	hemopexin	R.ELISER.W [82, 87]	x		
		K.VDGALCMEK.S [401, 409]	x		
	haptoglobin	K.DIAPTLTLYVGK.K [156, 167]	x		
		R.VMPICLPSK.D [202, 210]	x		
saliva	α amylase	R.WVDIALECER.Y [35, 44]			x
		K.SSDYFGNGR.V [258, 266]			x
	annexin 1	K.GVDEATIIDILTK.R [58, 70]			x
		R.SEIDMNDIK.A [303, 311]			x
		K.ILVALCGGN.- [337, 345]			x
	MUC 5AC	K.GVQLSDWR.D [1335, 1342]			x
		R.AQAQPGVPLR.E [2999, 3008]			x
	cystatin S	K.QLCSFEIYEVPWEDR.M [115, 129]			
		R.MSLVNSR.C [130, 136]			x
semen	MUC 6	K.VTNEFVSEEGK.F [182, 192]		x	
		R.ETDPCSMSQLNK.V [573, 584]		x	
		R.GVLLWGWR.S [646, 653]		x	
		K.VYHLPYYEACVR.D [1075, 1086]		x	
	SEM II	K.GHYQNVVDVR.E [217, 226]		x	
		K.DIFTTQDELLVYNK.N [251, 264]			
		K.ISYQSSSTEER.H [345, 355]		x	
		K.QDLLSHEQK.G [534, 542]		x	
	PSA	R.IVGGWECEK.H [20, 28]		x	
		K.HSQPWQVLVASR.G [29, 40]		x	
		R.LSEPAELTDAVK.V [121, 132]		x	
		K.FMLCAGR.W [190, 196]		x	
	PAP	R.SPIDTFPTDPIK.E [47, 58]		x	
		K.DFIATLGK.L [185, 192]		x	
		R.ELSELSLLSLYGIHK.Q [236, 250]		x	
	SEM 1	R.LWVHGLSK.E [165, 172]		x	
		K.VQTSLCPAHQDK.L [233, 244]		x	
		K.DVSQSSIYSQTEEK.A [307, 320]			
		K.GESGQSTNR.E [404, 412]		x	
urine	osteopontin	K.QNLLAPQTLPSK.S [51, 62]			
		R.GDSVVYGLR.S [145, 153]			
		R.ISHELDSASSEV.- [287, 298]			
	uromodulin	R.STEYGEGYACDTDLR.G [185, 199]			
		K.VFMYLSDSR.C [356, 364]			
		K.INFACSYPLDMK.V [420, 431]			
		R.VGGTGMFTVR.M [449, 458]			
		R.VLNLGPITR.K [597, 605]			

[a]X is reported when the unique peptides belonging to the target proteins characteristic of individual biological matrix were identified by MRM analysis.

discriminate among the different fluids, even in mixture by a single MRM run of less than 1 h.

Indeed, four proteins, α-globin, β-globin, haptoglobin, and hemopexin have been selected for blood signature, four for saliva (α-amylase 1, mucin-5, annexin A1, and cystatin S), five for semen (semenogelin I and II, PSA, PAP, and mucin-6) and two proteins determined for urine (uromodulin and osteopontin). For each protein biomarker, a number of unique tryptic peptides (proteotypics) has been selected with the use of bioinformatics tools and the best precursor ion-fragments transitions employed to set up a single MRM method able to identify any of the four biofluids in unknown stains. The MRM LC-MS/MS analysis recognizes the specific peptides by their unique transitions. The identified proteotypic peptides have been correlated to each set of selected protein biomarkers that in turn unambiguously defined the biological fluids under investigation.

The MRM method optimized on pure samples of the four matrices, blood, urine, semen and saliva, is resulted to have good specificity and selectivity for each biofluid with no cross-contamination observed whatsoever. A single contamination was detected in the urine samples spotted on different surfaces that showed the occurrence of small amount of seminal fluid.

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However, this presence was somehow expected and contributed to indicate a male donor. Then in a proof of concept, prepared test samples consisting of different fluids spotted on various substrates have been analyzed by using the single optimized MRM method.

The current results demonstrated that the developed strategy based on the MRM LC-MS/MS method could be a useful substrate in forensic science due to its capability of providing a universal approach for the identification of unknown stains recovered at crime scenes with high selectivity and specificity. Moreover, this approach can be carried out on the same samples used for DNA profiling revealing the nature of the tissue or fluid the DNA had been recovered from.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04742.

List of proteins identified for each biological matrix by different pretreatment by LC-MS/MS analysis; list of proteins selected as representative for each biological matrix by matching LC-MS/MS analysis and literature data; MRM method reporting selected peptide sequence, m/z precursor, m/z product ions and collision energy (V) optimized for a single biological fluid and the unique MRM method able to detect the type of fluid from unknown biological evidence on the crime scene; specificity test of MRM method when biological fluids were mixed on cloth; total ion current chromatogram from LC-MSMS analysis and the MRM chromatograms analysis of recorded transition for selected peptides for highly specific and selective identification of each biological fluid; and DNA profiling obtained by multiple amplification of 17 polymorphic regions (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: anna.illiano@unina.it.

ORCID

Anna Illiano: 0000-0003-1491-8966

Notes

The authors declare no competing financial interest.

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