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# Direct identification of forensic body fluids using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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ABSTRACT

The examination of body fluids such as blood, seminal fluid, urine or saliva is a key aspect of forensic science in regards to investigation of crimes against the person such as murder and rape. This article describes a streamlined and simplified approach for the identification of body fluids using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) that avoids fractionation or isolation of proteins. It is applicable to the examination of microliter quantities (or less) of neat fluids, extracts or suspensions of dried fluids or deposits of fluids in situ on tufts of fibers plucked from fabrics, which has practical application in the examination of crime-related items such as underwear or face masks. Hemoglobin was used as a marker for blood, α-amylase for saliva, semenogelins, prostate-specific antigen and acid phosphatase for seminal fluid and uromodulin for urine. Aged stains were successfully analyzed; blood proteins were easily detected in an 11-year-old blood stain.



## 1. Introduction

The examination of body fluids such as blood, seminal fluid, urine or saliva is a key aspect of forensic science in regards to crimes against the person such as murder and rape. The main test carried out on fluid deposits is DNA profiling in order to establish from whom the body fluid originated. However, it can be extremely important in a case, sometimes even crucially important, to establish the identity of the fluid(s) in the crime deposit in order to establish a link between a body fluid and the resulting DNA profile. Body fluids can be encountered in crime investigation as mixtures, for example blood and semen, semen and saliva, or mixtures containing urine. Several presumptive tests for body fluids are available but they can be ambiguous or provide false negative or false positive results, especially in the case of mixed deposits. Chemical tests (such as the luminol, Kastle–Meyer, leucomalachite green, and benzidine tests [1,2]) have been used for over 40 years as presumptive tests for blood and more recently medical test kits such as Hemastix and Hematrace [3] have been brought into use. In regards to chemical tests and Hemastix, the detection is based upon chemical reactions catalyzed by the heme group (iron) present in hemoglobin within the blood, but cross reactivity with other materials such as bleach, saliva, and other animal and fruit/vegetable proteins (e.g., peroxidases) is known [4,5]. Hematrace kits, being based upon immunochromatography, are much more specific but can lead to false negatives when high concentrations of hemoglobin are present, give rise to false positives in the presence of blood from ferrets and higher primates other than humans, and rely upon the epitope (the target for the antibody) being intact. Furthermore, these presumptive tests cannot distinguish between related body fluids such as venous blood and menstrual blood. Ultraviolet (UV) light can be used to stimulate fluorescent emission from semen stains, which is not only useful in presumptively indicating the presence of semen but it can be used by investigators to search a crime scene for invisible semen stains. However, emission can be quenched by various domestic substances or swamped by emission from other fluorophores present in items such as clothing [6]. The

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prostatic acid phosphatase (AP) test [7], which was developed in the late 1950s, has been reported to give some false positive color changes with plant material (e.g., tea plants [8]), and substances found within vaginal fluids and female urine as well as semen [9]. This could lead to confusion when identifying the body fluids of rape cases, where identifying fluid samples must indicate whether sexual intercourse had occurred or not. In addition, enzymes can degrade when exposed to environmental factors such as heat, mold, putrefaction, or denaturing chemicals. Immunochromatographic tests for the detection of semenogelin, prostate specific antigen (PSA), and prostatic acid phosphatase are now available [10–14]. For saliva, the most popular and simple test that is widely used in forensic laboratories for the detection of salivary α-amylase is the Phadebas assay [15]. The presence of α-amylase enzyme in a saliva sample or stain hydrolyses α-1-4 glycosidic bonds of a dye-labeled starch impregnated in a test paper, releasing the dye that forms a visible blue stain in the paper. However, it has been shown that false positive results are produced by hand cream, face lotion, urine and feces [16]. Immunochromatographic tests also are available for saliva, but false positive salivary amylase results have been reported for certain citrus fruits (the calamondin, or cumquat, [17]). Urine is another body fluid encountered in sexual assault, rape or murder, harassment and/or mischief cases. The traditional method for identifying urine is based on the detection of urea or urea nitrogen [18]. Urine is difficult to detect due to the low sensitivity of urea tests and many false positive results can be expected from this assay due to the ubiquity of urea. Previous studies [19] presented a method for indication of human urine by detection of five major 17-ketosteroid conjugates using HPLC–MS analysis. Another method relies upon the reaction between urine and a urease enzyme to form ammonia that in turn causes a color change in bromothymol blue indicator [20]. However, semen and sweat stains also display weak false positive results because of their urea content. The Tamm–Horsfall glycoprotein (THP), also known as uromodulin, has been used as a target in immunochromatographic tests (RSID™) for urine, but it is not a human-specific test [21].

While current forensic practices in regards to the identification of some body fluids using immunochromatographic tests are quite reliable, there are some significant shortcomings including: a shortage of immunochromatographic tests for a number of body fluids of forensic interest; the need to verify presumptive tests with confirmatory tests; and, as the presumptive tests respond to a single fluid, the requirement for a combination of tests in order to fully characterize a fluid or mixture. For these reasons, alternative ways to identify body fluids for forensic purposes are being sought.

Recently, research articles have been published that describe the use of mass spectrometry-based techniques for the identification of protein biomarkers for forensic purposes in biological fluids including hemoglobin in blood, α-amylase in saliva, and PSA and semenogelin protein markers in semen [22–25]. It has been shown that mass spectrometry-based techniques can detect body fluid biomarker proteins accurately, reproducibly and with high sensitivity. Identification is at the molecular level (i.e., protein sequence level), is species-specific and multiple markers for each type of body fluid can be used to increase the confidence of identification.

In 2013, Yang et al. used liquid chromatography–matrix-assisted laser desorption/ionization time of flight mass spectrometry (LC–MALDI-ToF-MS) for the identification of protein markers in different body fluids [22]. Another recently published article [24] describes a specific protein marker approach using comparative proteome fractionation followed by liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) analysis. The authors identified a panel of 29 candidate protein markers and suggested specific indicators of human urine, vaginal fluid, peripheral blood and menstrual blood [24]. These “classical” proteomics methods can play a significant role in the biological mass spectrometry for biomarker discovery in body fluids for forensic purposes. However, these technologies rely on some level of fractionation of the complex mixture of proteins, are time consuming, complex and bear the risk of column carry over between samples [26]. In other recent work, which extended an on-going theme of employing MALDI for the direct detection of endogenous and exogenous small molecules in fingermarks, it was indicated that MALDI can be used for the direct detection of blood proteins in fingermarks [25].

The aim of the research described in this paper was to extend and simplify MS-based identification of protein biomarkers and develop a methodology that is appropriate and practical for use in forensic casework. Here, we present a direct methodology for the positive identification of a broad range of body fluids of interest to forensic science (e.g., blood, semen, saliva and urine), including mixtures, using MALDI-TOF MS. In this study, we have used two different MALDI sample preparation strategies; the particular method chosen depended upon the nature of the evidence under examination. In the first strategy, untreated aliquots of liquid fluids or extracts or suspensions of dried flakes of them (such as would be found on non-porous surfaces) can be analyzed. In the second strategy, which can be applied to deposits on porous evidence such as garments or swabs, we present a novel and elegant approach that simply involves plucking single fibers (or a small tuft of them) from the fabric and directly analysing the body fluid deposits in situ. Two different ionization substrates, Bruker MTP polished steel plate and indium tin oxide (ITO) coated glass slides, were used depending upon which sample preparation strategy was chosen. We then combine these two sampling strategies with both “top-down” proteomic analysis (which is a rapid process ideal for screening of samples) and bottom-up analysis (which is used as a confirmation step).

## 2. Experimental

### 2.1. Materials

α-Cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), dithiothreitol (DTT), iodoacetamide (IAM), LC–MS grade water, acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (NSW, Australia). Trypsin gold (MS grade) was obtained from Promega (Madison, WI, USA). Indium-tin oxide (ITO) coated slides were purchased from Shimadzu (NSW, Australia). Polished steel plate (MTP) and protein/peptide mixture of external calibrants was obtained from Bruker Daltonics (Bremen, Germany).

Pieces of 100% nylon, cotton and cellulose acetate fabric (all white) were purchased from Spotlight (Adelaide, South Australia) and were laundered in lukewarm water for 30 min using non-enzymatic laundry powder (“Fab” brand, Colgate-Palmolive, Pty Ltd.) at the level recommended by the manufacturer. The fabric pieces were then rinsed several times in cold water and left to dry naturally.

Body fluid samples were sourced from eight volunteers from FSSA and Flinders University and semen samples were sourced from six patient volunteers from Flinders Fertility, Adelaide, South Australia pursuant to Southern Adelaide Clinical Human Research Ethics Committee Application 440.14 – HREC/14/SAC/455. Four proficiency test kits provided by Collaborative Testing Services (CTS) Incorporated (Stirling, Virginia, USA) were used. The tests used were 04-571 (provided by CTS in January 2004), 11-573 (May 2011), 12-584 (July 2012) and 14-582 (March 2014), which were provided to Forensic Science SA (FSSA, Adelaide, South Australia) as part of their quality assurance program. Each test involved four pieces of fabric stained with various body fluids; they were manufactured to resemble typical stained items that might be