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**DOI**

[10.3390/app14156657](https://doi.org/10.3390/app14156657)

**Publication date**

2024

**Document Version**

Final published version

**Published in**

Applied Sciences

**License**

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[Link to publication](#)

**Citation for published version (APA):**

Achetib, N., Otto, R. E., Aalders, M. C. G., & van Dam, A. (2024). Oxidative Modifications of Proteins and Lipids of Dried Semen, Urine, and Saliva Stains as a Function of Age in Forensic Context. *Applied Sciences*, 14(15), Article 6657. <https://doi.org/10.3390/app14156657>

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## Article

# Oxidative Modifications of Proteins and Lipids of Dried Semen, Urine, and Saliva Stains as a Function of Age in Forensic Context

Nihad Achetib <sup>1,2,†</sup> , Rosa E. Otto <sup>1,†</sup>, Maurice C. G. Aalders <sup>1,2,3</sup>  and Annemieke van Dam <sup>1,2,4,\*</sup> 

<sup>1</sup> Department Biomedical Engineering and Physics, Location AMC, Amsterdam University Medical Centers (UMC), University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; n.achetib@amsterdamumc.nl (N.A.); m.c.aalders@amsterdamumc.nl (M.C.G.A.)

<sup>2</sup> Methodology Research Program, Amsterdam Public Health Research Institute, Amsterdam University Medical Centers (UMC), Location AMC, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

<sup>3</sup> Co van Ledden Hulsebosch Center (CLHC), University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands

<sup>4</sup> Department Forensic Science, Amsterdam University of Applied Science, Tafelbergweg 51, 1105 BD Amsterdam, The Netherlands

\* Correspondence: annemiekevandam@amsterdamumc.nl; Tel.: +31-20-566-4390

† These authors contributed equally to this work.

**Abstract:** Knowledge of the time of deposition is pivotal in forensic investigations. Recent studies show that changes in intrinsic fluorescence over time can be used to estimate the age of body fluids. These changes have been attributed to oxidative modifications caused by protein–lipid interactions. This pilot study aims to explore the impact of these modifications on body fluid fluorescence, enhancing the protein–lipid model system for age estimation. Lipid and protein oxidation markers, including protein carbonyls, dityrosine, advanced glycation end-products (AGEs), malondialdehyde (MDA), and 4-hydroxynonenal (HNE), were studied in aging semen, urine, and saliva over 21 days. Surface plasmon resonance imaging (SPRi), enzyme-linked immunosorbent assay (ELISA), and fluorescence spectroscopy were applied. Successful detection of AGE, dityrosine, MDA, and HNE occurred in semen and saliva via SPRi, while only dityrosine was detected in urine. Protein carbonyls were measured in all body fluids, but only in saliva was a significant increase observed over time. Additionally, protein fluorescence loss and fluorescent oxidation product formation were assessed, showing significant decreases in semen and saliva, but not in urine. Although optimization is needed for accurate quantification, this study reveals detectable markers for protein and lipid oxidation in aging body fluids, warranting further investigation.

**Keywords:** oxidation markers; time of deposition; surface plasmon resonance imaging (SPRi); enzyme-linked immunosorbent assay (ELISA); fluorescence spectroscopy



**Citation:** Achetib, N.; Otto, R.E.; Aalders, M.C.G.; van Dam, A. Oxidative Modifications of Proteins and Lipids of Dried Semen, Urine, and Saliva Stains as a Function of Age in Forensic Context. *Appl. Sci.* **2024**, *14*, 6657. <https://doi.org/10.3390/app14156657>

Academic Editors: Ionut Relu Andrei and Mihai Boni

Received: 12 June 2024

Revised: 18 July 2024

Accepted: 26 July 2024

Published: 30 July 2024



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## 1. Introduction

Body fluids are complex biological fluids that contain proteins and lipids, which are highly susceptible to oxidation processes. When body fluids age, they can undergo a variety of oxidation reactions which can lead to changes in their chemical structure and physical properties. Oxidation reactions are known to occur during ageing, food processing, and storage and are associated with a number of diseases [1–3]. In the forensic field, several studies have been conducted to investigate the oxidative degradation of fingerprint residues [4–9] and to develop methods that rely on oxidation reactions to determine the time of deposition of biological evidence. Bremmer et al. developed an age estimation method for blood stains, based on the quantification of oxidation products of hemoglobin using reflection spectroscopy [10]. Van Dam et al. monitored protein–lipid reactions over time and used protein fluorescence and fluorescent oxidation products

(FOX) signatures to determine the age of fingermarks [11]. Achetib et al. showed that this fluorescence-based method is also applicable to other protein/lipid-containing traces, such as semen stains [12].

In foods, the oxidative reaction of proteins and lipids have been widely studied. The production of fluorescence due to protein–lipid interactions can occur through different pathways. Well-known oxidation reactions that induce fluorescent changes include the formation of protein carbonyls, dityrosine, and advanced glycation end-products (AGEs) [13–15]. Carbonyls can be formed due to protein–lipid reactions, whereby the lipid oxidation degradation products react directly with amino groups of amino acids leading to fluorescent compounds [16]. Dityrosine, the oxidized form of tyrosine, is a highly fluorescent molecule which is formed when a cross-link between two tyrosine residues is produced by a radical reaction. Fluorescence can also be produced in a non-radical reaction in which aldehydes react directly with amino groups of amino acids. Aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), play an important role in the degradation of amino acids and are considered important markers for lipid peroxidation (LP) [17]. Furthermore, MDA and HNE can lead to the formation of AGEs by a Maillard reaction. The Maillard reaction occurs when amino acids react with the carbonyl group of reducing sugars, such as glucose or aldehydes, and form Schiff bases. The base can rearrange itself to a stable Amadori product, which can undergo further rearrangement, cleavage, and covalent binding reactions forming AGEs [18]. It is thought that, as body fluids age, the presence of oxygen causes the unsaturated lipids to oxidize and form reactive oxidation products, which react with proteins to form FOX [11,12]. This ageing process shows similarities to the oxidation reactions that have been widely studied as they play a role in food quality assessment. We expect similar reactions to be involved in the formation of fluorescent protein–lipid complexes in ageing body fluids. Knowledge about the chemical reaction can improve the current protein–lipid model system upon which fluorescence-based age estimation methods rely. Therefore, the aim of the present study is to investigate if these lipid and protein oxidation markers can be measured in semen, saliva, and urine, over the course of time. The biomarkers will be measured in body fluid samples aged for 0, 2, 4, 7, 14, and 21 days, with a focus on the detection of protein carbonyls, dityrosine, AGEs, MDA, and HNE as oxidation biomarkers using surface plasmon resonance imaging (SPRi), enzyme-linked immunosorbent assay (ELISA), and fluorescence spectroscopy.

SPRi is a detection technique used to monitor biomolecular interactions in real time without the need of labels. The technique is based on the principle of surface plasmon resonance (SPR), which involves detecting changes in the refractive index near the surface of a sensor chip when target molecules bind to immobilized capture molecules on the chip [19–21]. The changes in the refractive index (RI) can be monitored over time with SPRi for different ligands simultaneously and various samples consecutively [22]. Images of the charge-coupled device (CCD) camera of the sensor surface provide detailed information on biomolecular interactions, such as affinity and kinetics. Since the detection of protein carbonyls requires a derivatization procedure of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) to form dinitrophenyl (DNP) hydrazine products, an ELISA assay was used. ELISA is an immunological assay designed for detecting and quantifying soluble substances such as antibodies, antigens, and proteins in biological samples [23]. In addition, the fluorescence spectra of the body fluids will be measured over time using fluorescence spectroscopy as previously performed by Van Dam et al. and Achetib et al. [11,12].

## 2. Materials and Methods

### 2.1. Sample Collection and Preparation

Before sample collection, informed consents were obtained from all volunteers and the experiments were conducted according to The Code of Conduct for Research Integrity of our country and our institutional guidelines. Further details of the collection procedure

can be found in Table 1. To monitor the dynamics of the body fluids over time, each vortexed sample was divided into small volumes and distributed over eight glass slides (Thermo Fisher Scientific, Waltham, MA, USA). Using small volumes ensures that the stains dry uniformly, as large stains may dry more slowly and potentially affect the oxidation processes. The eight samples were pooled to one sample for each donor at each time point, to ensure that the same amount of proteins and lipids were present in every sample. For time interval  $t = 0$ , the samples were processed after one hour. An amount of 35  $\mu\text{L}$  of semen was pipetted on each glass slide, while for urine and saliva 50  $\mu\text{L}$  was pipetted. In total, six time points were included:  $t = 0$ ,  $t = 2$ ,  $t = 4$ ,  $t = 7$ ,  $t = 14$ , and  $t = 21$  days. The samples were left to age in a dark environment at room temperature until collection. The samples were collected at the set time intervals by scraping the body fluids off the glass slides using disposable razors. The scraped body fluid samples were placed into Eppendorf tubes (Eppendorf, Hamburg, Germany) and dissolved in phosphate buffer saline (PBS) (Westburg, Leusden, The Netherlands). The PBS volumes were 35, 75, and 50  $\mu\text{L}$  for semen, urine, and saliva, respectively. Finally, for each time interval the samples were pooled per donor to obtain a homogenous solution and then stored in the freezer at  $-80\text{ }^{\circ}\text{C}$  until the samples of each time point were collected. Before SPRi analyses, body fluid samples were diluted in PBS in the ratios: semen 1:10, urine 1:5, saliva 1:1.

**Table 1.** Details of the sample collection procedure of semen, urine, and saliva. \* M represents male volunteers and F female volunteers.

Biological Trace	Gender (M/F) *	Place of Donation	Storage	Collection Procedure	Additional Information
Semen	10 M	Fertility clinic of Isala Hospital	At $-20\text{ }^{\circ}\text{C}$ ( $n = 3$ ) At $-80\text{ }^{\circ}\text{C}$ ( $n = 7$ )	Collected in sterile collection cups (Sarstedt, Germany).	None.
Urine	10 F	Amsterdam University Medical Centers	No storage	Collected in the morning in sterile collection cups (Sarstedt, Germany). Volunteers were instructed not to collect the first morning urine of the day but the second or later.	An amount of 6/10 urine samples remained available for further analysis to confirm their health status. Protein, blood, leucocytes, nitrite, glucose, ketone, pH, bilirubin, and urobilinogen (Siemens Healthcare Diagnostics, Erlangen, Germany) were analyzed and no abnormalities were found.
Saliva	10 F	Amsterdam University Medical Centers	No storage	Collection was conducted in the morning. Instruction to not consume any food/drinks for at least half an hour prior donation. A saliva collector (Oracol, Malvern Medical Developments Limited, Worcester, UK) was used for 1 min to stimulate the saliva production.	None.

## 2.2. Antibodies and Sensor Spotting

Antibodies (Table 2) specific for the oxidation markers MDA, HNE, AGEs, and di-tyrosine were selected. In addition, antibodies for body fluid-specific markers and isotypes (Table 2) were included as controls [22]. The body fluid-specific markers included semenogelin-1 (SEM) and prostate-specific antigen (PSA) for semen, Tamm–Horsfall protein (THP) and osteopontin for urine, and alpha-amylase 1 (AMY-1) for saliva. Coupling buffer (CB) was used as the immobilization buffer to obtain a concentration of 5  $\mu\text{g}/\text{mL}$

of antibody immobilized on the sensor. Coupling buffer consisted of 19 mmol/L sodium acetate and 31 mmol/L acetic acid (both Merck, Darmstadt, Germany) supplemented with 0.05% (*v/v*) Tween 20 (Sigma-Aldrich, Darmstadt, Germany), pH~4.5. Only polyclonal anti-MDA and THP were diluted to 10 µg/mL and 2.5 µg/mL, respectively.

**Table 2.** Overview showing the selected antibodies and respective isotypes that were used.

	Antibody	Isotype	Commercial Concentration µg/mL	Manufacture
<b>Oxidation biomarkers</b>	Anti-Malondialdehyde	Mouse monoclonal IgG1	1000	Abcam, Cambridge, UK
	Anti-Malondialdehyde	Rabbit polyclonal IgG	1000	Cloud-Clone Corp., Wuhan, China
	Anti-4-hydroxynonenal	Rabbit polyclonal IgG	500	Cel biolabs Inc., San Diego, CA, USA
	Anti-4-hydroxynonenal	Goat polyclonal IgG	1000	Cel biolabs Inc., San Diego, CA, USA
	Anti-dityrosine	Mouse monoclonal IgG2a/k	200	Japan institute for the controls of aging, Fukuroi, Shizuoka, Japan
	Anti-Advanced glycation end product	Mouse monoclonal IgG1/k	1000	Cloud-Clone Corp., Wuhan, China
<b>Body fluid-specific biomarkers</b>	Anti-semenogelin-1	Mouse monoclonal IgG1	200	Santa Cruz, CA, USA
	Anti-human-prostate-specific-antigen	Mouse monoclonal IgG1	1000	Bio-Rad, Hercules, CA, USA
	Anti-human Tamm–Horsfall ascites	Mouse monoclonal IgG2b	200	Cedarlane, Burlington, ON, USA.
	Anti-osteopontin	Mouse monoclonal IgG1/k	1000	Biovision, Milpitas, CA, USA
	Anti-alpha amylase 1	Mouse monoclonal IgG2a/k	400	Abgent, San Diego, CA, USA
<b>Isotype controls</b>	IgG Isotype control	Rabbit polyclonal IgG	1000	Merck KGaA, Darmstadt, Germany
	IgG1 Isotype control	Mouse monoclonal IgG1	500	R&D systems, Minneapolis, MN, USA
	IgG2a Isotype control	Mouse monoclonal IgG2a	500	R&D systems, Minneapolis, MN, USA
	IgG2b Isotype control	Mouse monoclonal IgG2b	200	Thermo Fisher Scientific, Camarillo, CA, USA

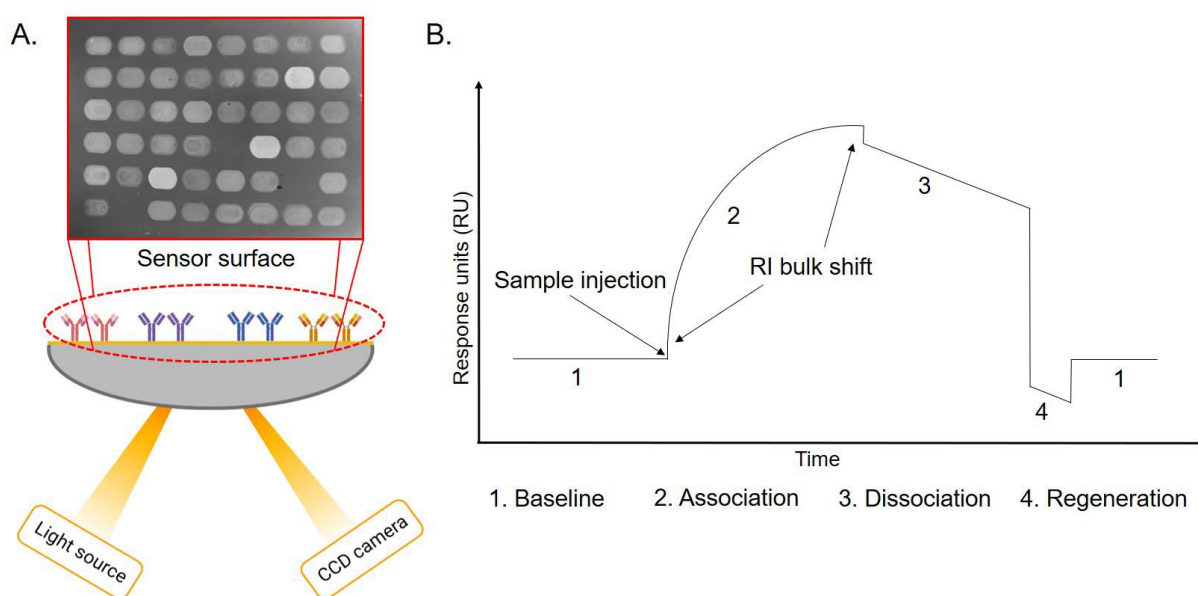
The diluted antibodies were printed with the Continuous Flow Microspotter (CFM) 2.0 (Wasatch Microfluidics LLC, Salt Lake City, UT, USA) onto the sensor. The sensor surface (Easy2Spot type-G, Ssens bv., Enschede, The Netherlands) contains a conducting gold layer and a 3D hydrogel-like layer which allows covalent binding of the antibodies to the sensor. The antibodies for the oxidation markers, body fluid-specific markers, isotypes, and coupling buffer (negative control) were printed in triplicate, which resulted in 48 spots. When the immobilization of the antibodies on the sensor was completed, the sensor was directly transferred to the IBIS MX96 SPR Imager (IBIS technologies B.V., Enschede, The Netherlands).

### 2.3. SPRI Analysis

#### 2.3.1. SPR Measurement

The antibody-coated sensor surface was continuously illuminated by a fixed laser diode at a wavelength of 840 nm. The light passed through the prism, reflected on the back side of the sensor chip surface, and was captured by a CCD camera, which monitored the intensity of the reflected light over time for the regions of interest (ROIs); see Figure 1 for SPRi set-up. The images of the camera were visualized in real time using DAX software version 1 (IBIS technologies, Enschede, The Netherlands). Before analysis, the sensor was treated with two blocking buffers composed of 100 mmol/L 2-amino ethanol and 1%

(*v/v*) bovine serum albumin (BSA) (both Sigma-Aldrich, Darmstadt, Germany) to prevent non-specific binding to the sensor. The SPR measurement started with four regeneration runs, to wash and stabilize the sensor, followed by a sample run and ended with a single regeneration run before the injection of the next sample. Each cycle of the regeneration run consisted of three phases: (i) baseline phase of two minutes, (ii) association phase of 20 min with 120  $\mu\text{L}$  regeneration buffer (RB) (0.1M Glycine,  $\text{pH} \pm 3.0$  (Merck, Darmstadt, Germany) and 0.05% Tween-20 (*v/v*) (Sigma-Aldrich, Darmstadt, Germany)), and (iii) dissociation phase of 8 min to wash the sensor with 240  $\mu\text{L}$  PBS. For the sample run, the same cycle was repeated, but during the association step, 150  $\mu\text{L}$  body fluid was injected instead of regeneration buffer. The sample was flowed back and forth over the sensor surface, enabling specific binding of the biomarkers in the sample to the immobilized antibodies. Subsequently, a single regeneration run was performed to remove the bound antigens to reverse the interaction between the antigens and immobilized antibodies and to return the response signals to baseline.



**Figure 1.** Schematic experimental set-up for SPRi. (A) Gold-plated sensor chip with immobilized antibodies on a prism. Upon illumination with P-polarized light from different angles, SPR causes a dip in the intensity of the reflected light at the incident angle or the so-called SPR angle, which is recorded by a CCD camera. Refractive index changes are caused by the binding of biomarkers with the antibodies. (B) The procedure starts with the injection of PBS to create a baseline (1), followed by flowing the body fluids over the sensor to bind the antigens with the specific antibodies on the surface (2., association phase). To remove unbound antigens, PBS is flowed again over the sensor (3., dissociation phase). Subsequently, a regeneration buffer is added to remove all bound antigens (4), to allow a new sample to bind to the antibodies.

### 2.3.2. SPRi Data Processing

The changes in RI were continuously monitored by the DAX software version 1 and converted into response units (RUs) (1 RU represents a RI change of  $1 \times 10^{-6}$ ). For the ROIs, the changing RUs were plotted over time, creating sensorgrams. The SPR-generated trix files were converted to ibmx files using SprintX software version 2.4.1.2 (IBIS Technologies, Enschede, The Netherlands) and an in-house-generated Matlab script was used in order to allow data analysis. To increase the quality of the experimental results, the local ligand density response (RLL) values were evaluated. Spots with large deviations in ligand density between triplicates were excluded. Subsequently, all sensorgrams were subjected to two signal modification steps, namely zeroing and local referencing. Zeroing was performed to correct for spot-to-spot variation and was achieved by setting the SPR signals to zero at the

start of the initial baseline. Local referencing was performed to correct for background noise and was realized by subtracting the SPRi signals of reference spots from the signals of the ROIs. After the corrections, the mean SPRi signal between 60 and 70 s of the baseline was subtracted from the mean SPRi signal between 120 and 130 s of the dissociation phase to calculate the biomarker quantities. The first minute of the baseline and the second minute of the dissociation phase were excluded to guarantee the removal of signals induced by bulk shift changes, which are the result of RI differences between the buffers. When the calculated response resulted in a negative value, the value was set to 0. A negative response could be the result of a higher signal of the reference spot compared to the antibody spot, or because the baseline response was higher than the dissociation response. Since the antibodies were printed in triplicate, the mean response signal and standard deviation were calculated to determine the mean response signal per biomarker. To ensure data quality and reliable results, samples with negative response signals were excluded when the triplicate responses of a single biomarker were all three negative and/or when all the body fluid-specific markers provided negative responses. Negative signals imply that the samples were too diluted or that the signals fall within the range of instrumental noise. The biomarkers were considered present in the body fluids when the positive responses signals were  $>0$ .

#### 2.4. Protein and Lipid Concentrations

The total protein content present in the body fluids was determined with a Pierce BCA Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Concentration of the protein carbonyls were determined using the protein carbonyl ELISA kit (Enzo Life Sciences, Faringdale, NY, USA) and the absorbances were measured at 450 nm with a microplate reader (BioTek ELx808 Absorbance Microplate Reader, Bio-Tek Instruments, Inc., Charlotte, VT, USA). For the protein carbonyls, a Shapiro–Wilk test was conducted to test for normality followed by one-way ANOVA to determine if significant changes occur over time for the body fluids.

#### 2.5. Fluorescence Spectroscopy

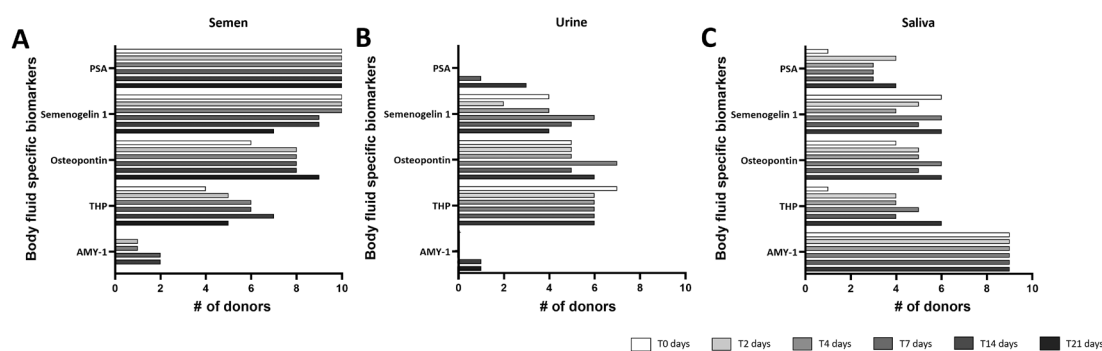
From the pooled samples which were described in Section 2.1, “Sample collection and preparation”, six spots of 2  $\mu$ L sample were pipetted on a thin layer chromatography plate (Silica gel 60, Merck, Germany) as fluorescent measurements were obtained at six different times after deposition. Fluorescence measurements were acquired using a LS55 Luminescence spectrometer equipped with a fiber optic accessory (Perkin Elmer, Waltham, MA, USA). To obtain the protein fluorescence, the samples were excited at 283 nm, while the emission was recorded at 313 to 550 nm. The fluorescence of the FOX was recorded at an excitation wavelength of 365 nm, in the emission wavelength range from 400–500 nm. For measuring semen, the (physical) slit width in the light path at the excitation and emission side of the LS55 resulted in spectral resolutions of 10 and 7.5 nm, respectively. For urine, both the excitation and emission spectral bandwidth were 15 nm, while for saliva, they were 10 and 5 nm, respectively. Data analysis included background correction and the calculation of the protein/FOX ratios by dividing the average maximum emission intensities of these spectra using Matlab software R2023b. For semen and saliva, the fluorescence intensity at 340 nm was divided by the fluorescence intensity at 430 nm. For urine, the fluorescence intensity at 370 nm was divided by the fluorescence intensity at 430 nm. The protein/FOX ratios were calculated for every time point of each time series. For one urine donor and two saliva donors, the samples were excluded due to very low FOX levels.

### 3. Results

#### 3.1. Detection of Body Fluid-Specific Markers Using SPRi

Body fluid-specific markers were included as control, to confirm that the extraction of the body fluids from the glass slides was conducted successfully. PSA and semenogelin-1, which are known proteins that are highly abundant in semen traces, could be detected

in ten out of ten fresh samples and ten out of ten fresh samples, respectively (Figure 2A). However, the number of samples in which semenogelin-1 could be detected decreased over time to seven out of ten samples after 21 days. For urine, three samples were excluded as these samples did not pass our inclusion criteria, which are described Section 2.3.2, “SPRi data processing”. The body fluid-specific markers osteopontin and THP for urine could be measured in at least five out of seven urine samples for all time points (Figure 2B). Amylase, a highly abundant enzyme in saliva, could be detected in nine out of nine samples (Figure 2C), for all time intervals. Samples from one specific donor did not pass our inclusion criteria at all time intervals and were therefore excluded. The total number of donors in which non-specific body fluid biomarkers could be detected was high, but expected since these biomarkers are present in multiple body fluids. PSA, for instance, is found in low concentrations in female urine, while amylase is present in urine and semen [24,25]. In urine and semen, amylase could be measured in two out of four and one out of seven, respectively. Additionally, because seminal fluid and urine travel through the same tract, traces of osteopontin and THP could be present in semen. Body fluids are complex samples, thus effects from matrix constituents or interfering agents to antibody sites could influence the response signals. To assess background levels, we used coupling buffer as negative control. Isotype controls were also included to assess non-specific binding of antibodies, ensuring that observed signals were specific to the target biomarkers. Future studies should analyze the specificity of the antibodies by including control samples with known concentrations of the biomarkers. Additionally, we performed replicate measurements to ensure reproducibility and consistency in our findings.

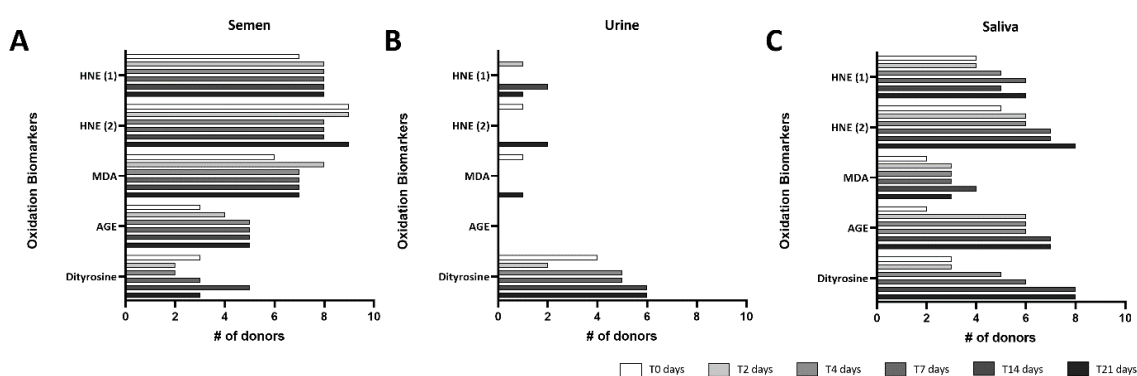


**Figure 2.** Overview of the number of samples in which body fluid-specific markers could be detected from semen, urine, and saliva samples. (A) Semen-specific markers were PSA and semenogelin-1. (B) Urine-specific markers were Osteopontin and THP. (C) Saliva-specific marker was AMY-1.

### 3.2. Detection of Oxidation Biomarkers in Body Fluids Using SPRi

All oxidation biomarkers were successfully detected in fresh and ageing semen and saliva (Figure 3). However, for urine, the lipid oxidation markers could not be detected. In semen, the LP marker HNE (1) could be measured for at least seven out of ten samples for all time points (Figure 3A) and HNE (2) could be measured in eight out of ten semen samples. The other LP marker, MDA, could be measured in at least six out of ten semen samples for all time points. The detection of LP markers in semen was expected to be successful as semen contains a large amount of polyunsaturated fatty acids (PUFAs), which are highly susceptible to oxidation [26]. The presence of the protein oxidation markers AGE and dityrosine appears to be low in semen samples (Figure 3A). The number of semen samples in which AGEs could be detected increased to five after four days and remained constant up to 21 days. The presence of dityrosine appears to be lower in semen samples, with the minimal number of samples (two) positive on days two and four and the maximum number of samples (five) at day 14. No clear pattern for the detection of dityrosine could be observed over time. With regard to urine, dityrosine could be detected in two urine samples (out of six) and over time increased to six samples (out of six) (Figure 2B). There can be multiple factors that can contribute to formation of dityrosine in urine including

oxidative stress, non-enzymatic mechanisms under certain conditions, such as exposure to UV light or metal-catalyzed oxidation, or the enzymatic action of peroxidases such as myeloperoxidase which are present in certain cells and can catalyze the cross linking of tyrosine residues [27–29]. AGEs could not be detected in any sample at each time point, while the LP markers HNE and MDA were measured in only one to two urine samples over time. With regard to saliva, all oxidation markers could be detected and the detection of the oxidation markers increased over time (Figure 3C). HNE showed the highest number of positive samples and could be measured in at least four out of nine samples. Over time, the number of samples increased in which HNE could be detected. Dityrosine could be measured in at least three out of nine fresh saliva samples and increased up to eight out of nine samples after 14 days. AGEs were detected in two out of nine fresh saliva samples but after 14 days increased to seven out of nine samples. MDA had the lowest number of positive samples in which it could be detected, varying from two fresh samples to four samples after 14 days and then reducing to three samples after 21 days.



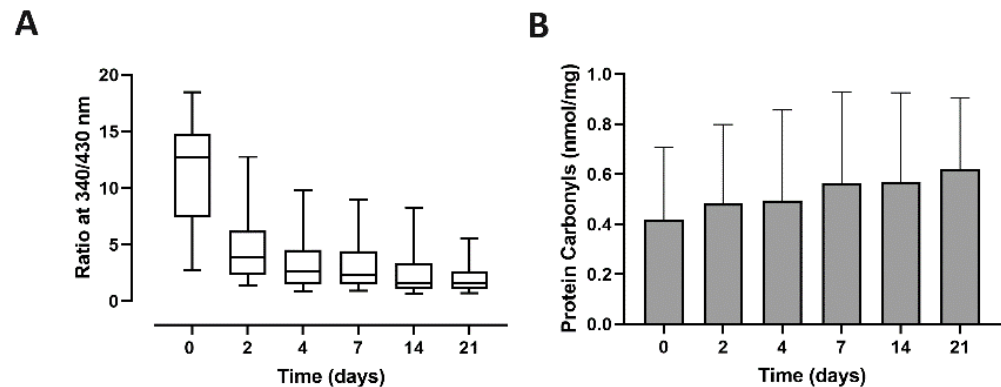
**Figure 3.** Overview of the number of donors in which oxidation markers could be detected from semen (A), urine (B), and saliva (C) samples.

### 3.3. Oxidation With Ageing

In addition to the measurement of the oxidation markers, fluorescence and protein carbonyls were measured in semen, urine, and saliva to determine whether there is evidence that oxidation plays an important role in ageing of body fluids. The ratios of protein and FOX were determined and protein carbonyl levels were measured, as it is evident that oxidation increases the amount of carbonyl groups [13,30]. The distribution of the fluorescent ratios and the protein carbonyl concentrations can be found in the Supporting Information.

#### 3.3.1. Semen

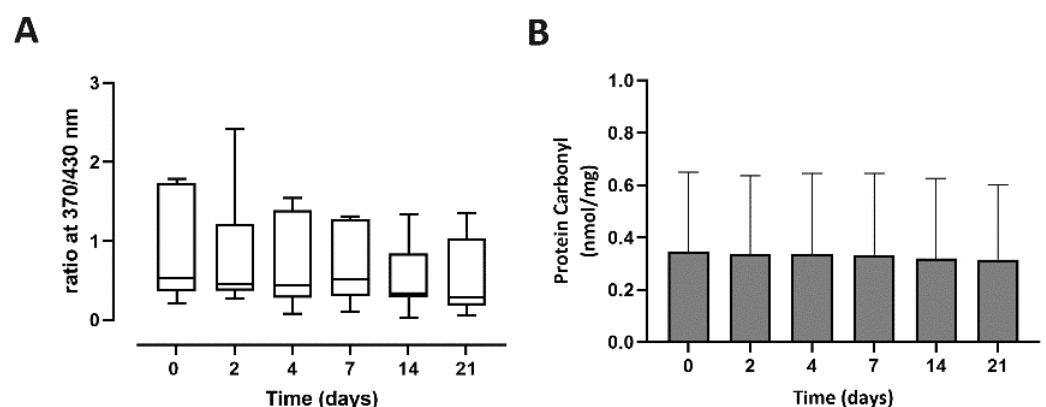
For protein and FOX fluorescence, the maximum fluorescence intensity is on average at a wavelength of 341 (SD 3.5) nm and 433 (SD 3.9) nm, respectively. As such, to determine the ratio, the fluorescence intensity at 340 nm was divided by the fluorescence intensity at 430 nm. Figure 4A illustrates the median ratio at 340/430 over time for semen samples. The fluorescence ratios of the semen samples decreased during oxidation, while the formation of carbonyls compounds increased (Figure 4B). There was a statistically significant decrease in the fluorescent ratios over time for semen (Figure 4A), ( $F(5,54) = 13.49$ ,  $p \leq 0.01$ ). A Tukey post hoc test revealed that fluorescence ratios were statistically significantly lower between day 0 and day 2 ( $6.84 \pm 1.3$ ,  $p \leq 0.001$ ), day 4 ( $8.23, 1.3$ ,  $p \leq 0.001$ ), day 7 ( $8.53 \pm 1.3$ ,  $p = 0.005$ ), day 14 ( $9.17 \pm 1.3$ ,  $p \leq 0.001$ ), and day 21 ( $9.5 \pm 1.3$ ,  $p \leq 0.001$ ). However, there was no statistically significant increase in the protein carbonyl levels over the course of time (Figure 4B), as determined by ANOVA ( $F(5,54) = 0.482$ ,  $p = 0.788$ ). Both figures show that there are large inter-donor differences between the samples.



**Figure 4.** Overview of oxidation parameters in semen during ageing. (A) Median protein/FOX ratio over time, with whiskers indicating the range from the smallest to the largest ratio. A significant decrease in the fluorescence ratios of semen over time is observed. (B) Mean protein carbonyl concentrations (nmol/mg) over time with error bars representing standard deviation. There was no statistically significant increase in protein carbonyl levels over time.

### 3.3.2. Urine

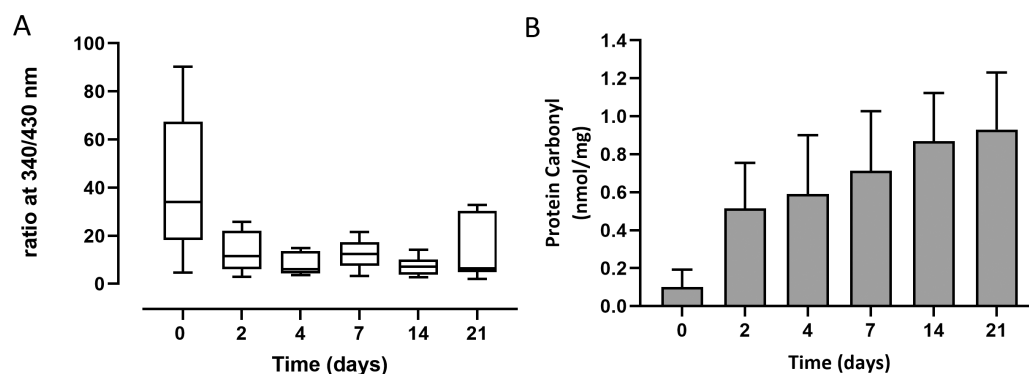
For urine, in contrast to semen and saliva samples, the average maximum fluorescence intensity of the protein emission spectra was found at 371.7 (SD 22.6) nm. The maximum intensity of the FOX peak was at approximately the same wavelength (431.4 (SD 8.5) nm) as the semen and saliva samples. Therefore, the ratio of the fluorescence intensities at wavelength 370 over 430 was plotted as a function of time. Although the median fluorescent ratios of urine decreased over time, the results were not found to be statistically significant (Figure 5A), ( $F(5,43) = 0.876, p = 0.505$ ). This was unexpected, as a previous study observed a decline in the fluorescence ratios of urine over time using the area under the curve (AUC) of the protein fluorescence at excitation 285 nm/emission 330–400 nm and the FOX fluorescence at excitation 365 nm/emission 400–500 nm [31]. In addition, the protein carbonyl levels seem to remain stable upon ageing and no statistically significant differences were observed over the course of time (Figure 5B), as determined by ANOVA ( $F(5,54) = 0.01, p = 0.999$ ). Again, both figures show large error bars, which indicate that the ratio and protein carbonyl levels vary between different donors.



**Figure 5.** Overview of oxidation parameters in urine during ageing. (A) Median protein/FOX ratio over time, with whiskers indicating the range from the smallest to the largest ratio. The median protein/FOX ratio decreased over time, but this change was not found to be statistically significant. (B) Mean protein carbonyl concentrations (nmol/mg) over time with error bars representing standard deviation. Note that the mean protein carbonyl concentration remains stable with ageing.

### 3.3.3. Saliva

The average maximum fluorescence intensity of protein and FOX for saliva were at the wavelengths 338 (SD 3.2) nm and 426 (SD 12.8) nm, respectively. The ratio of the fluorescence intensities at wavelength 340 over 430 was plotted as a function of time (Figure 6A). The median fluorescence ratios decreased over time ( $F_{(5,35)} = 5.412, p < 0.001$ ). A Tukey post hoc test revealed that fluorescence ratios were statistically significantly lower between day 0 and day 2 ( $28.18 \pm 7.5, p = 0.008$ ), day 4 ( $33.27 \pm 7.5, p = 0.001$ ), day 7 ( $28.5 \pm 7.3, p = 0.005$ ), day 14 ( $33.5 \pm 7.5, p = 0.001$ ), and day 21 ( $27.1 \pm 7.8, p = 0.016$ ). There was no statistically significant difference in the fluorescence ratios among the other time intervals. While the fluorescence ratios decreased over time, the protein carbonyl concentrations increased (Figure 6B), ( $F_{(5,54)} = 13, p = 0.001$ ). A Tukey post hoc test showed that the protein carbonyl concentration was statistically significantly lower for day 0 ( $0.100 \pm 0.091$  nmol/mg) compared to day 2 ( $0.515 \pm 0.240$  nmol/mg,  $p = 0.010$ ), day 4 ( $0.590 \pm 0.310$  nmol/mg,  $p = 0.001$ ), day 7 ( $0.714 \pm 0.313$  nmol/mg,  $p = 0.000$ ), day 14 ( $0.869 \pm 0.254$  nmol/mg,  $p = 0.000$ ), and day 21 ( $0.930 \pm 0.300$  nmol/mg,  $p = 0.000$ ). Furthermore, the protein carbonyl concentrations were also significantly lower on day 2 compared to day 14 ( $p = 0.043$ ) and day 21 ( $p = 0.011$ ).



**Figure 6.** Overview of oxidation parameters in saliva during ageing. (A) Median protein/FOX ratio over time, with whiskers indicating the range from the smallest to the largest ratio. (B) Mean protein carbonyl concentrations (nmol/mg) increases over time with error bars representing standard deviation. Note that The median protein/FOX ratio decreased over time, while the protein carbonyl concentrations (nmol/mg) increased.

## 4. Discussion

Chemical and physical properties of biological traces are directly affected by oxidative modifications of proteins and lipids. However, the influence of protein and lipid oxidation on the ageing of body fluids *ex vivo* is poorly understood. Currently, there is an increasing interest among forensic scientists to acquire knowledge about the ageing of body fluids for the development of age estimation methods [32]. As such, in the present pilot study, different parameters to assess protein and lipid oxidation were determined for the first time using fluorescence spectroscopy, ELISA, and SPR.

Fluorescence spectroscopy is commonly used in biomedical science to quantify protein oxidation. Viljanen et al. used fluorescence spectroscopy to evaluate the oxidation of bovine serum albumin (BSA) and dairy proteins [16]. Protein oxidation was determined by measuring the loss of the natural tryptophan fluorescence and the gain of protein carbonyls [16]. Similar kinetics were observed by van Dam and Achetib in ageing of fingerprints and semen, respectively [11,12]. However, since non-contact with the body fluid is necessary to allow the use of this technique in forensic practice, the researchers measured the fluorescence with a fiber-based accessory. To eliminate effects of height differences of the fiber between the time intervals, the ratio was used as measure of protein oxidation. The maximum fluorescence intensities of protein fluorescence over time were measured between 320–340 nm for semen and saliva and between 340–370 nm for urine.

The second peak, which represents the fluorescent oxidation products, was observed between 400–450 nm for semen and urine samples. For saliva, low fluorescence intensities could be observed at 430 nm. Low fluorescence of the fluorescence oxidation products was expected due to the low presence of PUFAs in saliva [33]. Saliva was the only body fluid for which a significant difference in the fluorescence ratios could be observed over time. The decrease in fluorescence ratios is in agreement with previous findings reported in the literature [31].

Carbonyl compounds are products of protein oxidation that are involved in cross-linking of damaged proteins via Schiff base formation [34]. Schiff bases can be formed when aldehydes, such as MDA and HNE, react with amino groups from the side chain of proteins. They can be detected around 450 nm upon excitation at 350 nm, which corresponds with the emission spectrum of our unknown fluorescent products. Protein carbonyls were detected in all three body fluids; however, only low concentrations (varying from  $0.14 \pm 0.06$  to  $0.892 \pm 0.02$  nmol/mg) of protein carbonyls could be detected in urine and therefore high concentrations of MDA, HNE, and AGE were not expected as these are involved in the formation of protein carbonyls. This is in agreement with our study results (Figure 3B), as we observed that these markers could only be detected in a few samples. We hypothesize that, as the fluorescence ratio decreases, carbonyl protein concentration increases. The protein carbonyl concentrations increased over time for semen and saliva, which indicates that protein carbonyl contributes to the emission spectrum of fluorescence oxidation products but may not be a key player. Only for saliva could a statistically significant difference be determined, but the use of a larger sample sizes may improve the statistical significance of these results. A significant difference could be used as a measure to distinguish between fresh and aged samples. However, it is clear that further research is needed to understand the changing dynamics in ageing body fluids. The presented results are preliminary and serve as proof of concept that protein and lipid oxidation markers are present in dried body fluids stains and that they change over time. The large variation observed between samples indicates that changes are donor-dependent, likely due to the initial composition of the stains. This observation is also seen when using fluorescence spectroscopy, where every sample has its own ageing kinetics. Future research should focus on increasing sample size, so the use of advanced statistical methods is possible to account for individual variability. Furthermore, we included urine and saliva samples exclusively from female donors. We acknowledge that including both sexes could provide more comprehensive insights and will enable us to examine potential sex-specific differences in protein and lipid oxidation in body fluids.

In this pilot study, we used SPRi to determine whether the oxidation markers were present in aged samples. However, to improve the sensitivity and specificity, mass spectrometry could be included, which also could lead to the identification of unique oxidation sites and predictive markers. Previously, Schneider et al. highlighted the potential of using advanced metabolomics profiling techniques to detect time-dependent changes in various body fluids. Their study demonstrated that while significant progress has been made in identifying molecular features associated with the time since deposition, challenges remain. Additionally, considering other influencing factors such as substrate, temperature, humidity, UV light exposure, and microbial activity is crucial for developing a robust forensic age estimation method.

## 5. Conclusions

In this pilot study, protein and lipid oxidation markers were detected and monitored for the first time in dried body fluid stains using SPRi, ELISA, and fluorescence spectroscopy. Important markers for LP and protein oxidation, including MDA, HNE, AGE, dityrosine, and protein carbonyls, were identified in semen, urine, and saliva. However, only in saliva was a significant decrease in fluorescence ratios and a significant increase in protein carbonyls observed over time. Our results provide insights about oxidation in ageing body fluids and highlight the need for more detailed investigation in this area.

Significant gaps remain in the understanding of oxidative modifications of proteins and lipids over time. More research is needed to establish a fundamental basis for understanding the ageing of biological fluids and to develop an accurate age estimation method for forensic investigations. Future research will focus on chemical reactions between proteins and lipids in dried body fluid stains, using mass spectrometry, immunogenic methods, and fluorescence spectroscopy to provide a comprehensive understanding of the ageing processes in a forensic context.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app14156657/s1>, Figure S1: Distribution of the fluorescent ratios and the protein carbonyls concentrations of each donor for semen, urine, and saliva.

**Author Contributions:** Conceptualization, N.A. and A.v.D.; Formal analysis, N.A. and R.E.O.; Funding acquisition, A.v.D.; Investigation, N.A. and R.E.O.; Methodology, N.A. and R.E.O.; Project administration, M.C.G.A. and A.v.D.; Software, N.A., R.E.O. and A.v.D.; Supervision, M.C.G.A. and A.v.D.; Visualization, N.A. and R.E.O.; Writing—original draft, N.A.; Writing—review and editing, N.A., R.E.O., M.C.G.A. and A.v.D. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research is granted by the Dutch Research Council (NWO), which is funded by the Ministry of Education, Culture and Science, project number: 18237.

**Institutional Review Board Statement:** Research protocols were performed in accordance with the Declaration of Helsinki and relevant guidelines and regulations of the Netherlands Code of Conduct for Research Integrity and the research code of the Academic Medical Center.

**Informed Consent Statement:** Informed written consents were obtained from all donors.

**Data Availability Statement:** The datasets generated and analyzed in the current study are available from the corresponding author on reasonable request.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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