# Evaluation of the effect of ozone disinfection on samples related to forensic medicine

Doctoral (PhD) thesis

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# Introduction

In the operation of a forensic genetics laboratory, the use of personal protective equipment (PPE) is essential both for the protection of the laboratory personnel and to prevent contamination of the samples to be analyzed. However, in addition to the routine protective equipment (gowns, rubber gloves, and disposable surgical masks), it is necessary in some cases to extend the list of precautions to be taken. This is particularly recommended when analyzing biological samples potentially contaminated with a pathogen. The presence of a microorganism that poses a risk to laboratory staff may be the result of an unintentional event, such as an epidemic, or the result of a deliberate act, like bioterrorism.

To prevent infection, alongside the use of higher-level PPE, we lack methods that can inactivate pathogens without damaging the samples to be analyzed. However, the necessity of such methods became evident during the COVID-19 pandemic. A quick and effective disinfection procedure that does not negatively impact either the environment or the sample residues would be desirable. The currently available UV- and chlorine-based disinfection methods not only damage the genetic material of the sample but also have other limitations, such as being time-consuming and ineffective in disinfecting porous and hard-to-reach places. Due to its gaseous state, ozone can overcome these latter difficulties, but we do not have sufficient knowledge about the consequences of its oxidizing effects on molecular biological methods.

The disinfecting properties of ozone were exploited for medicinal purposes as far back as 150 years ago for the treatment of infections and wounds. However, despite this long history, our knowledge about the exact mechanism of action and the potential applications of ozone treatment remains very limited to this day.

Ozone disinfection is a routinely utilized method for cleaning the air conditioning systems of vehicles, and during the COVID-19 pandemic, it gained even broader usage, becoming known and accessible to more people. My research focused on the applicability of ozone disinfection in the field of forensic genetics.

## Aims

In my research, I examined the effect of ozone disinfection on the most common types of samples encountered in forensics. Among body fluids, I investigated blood, saliva, and semen, as well as DNA deposited on inanimate objects that come into contact with the skin, following ozone treatment. The objectives of my research were as follows:

- Analyze the applicability of presumptive tests after ozone treatment.
- Evaluate the impact of ozone treatment on confirmatory tests, especially on the detectability of tissue-specific mRNA markers.
- Investigate the effect of ozone treatment on isolated DNA samples, regarding the success of STR profiling.
- Examine the STR profiles of body fluids after ozone treatment.
- Genetic analysis of samples deposited during shedding of epithelial cells after ozone treatment, evaluation of the success of STR profiling.

## Materials and methods

### Sample preparation

#### *Investigation of the effect of ozone treatment on body fluids*

To examine the effect of ozone treatment on body fluids, I pipetted 1 µl and 10 µl samples of blood, saliva, and semen onto clean, white cotton fabric. To simulate the small amounts of samples commonly encountered in forensic cases, I applied the 10 µl spots in a tenfold dilution series, ranging from undiluted to a 1:1000 dilution (**Table 1**). For microscopic examination of sperm cells, semen samples were pipetted directly onto sterile microscope slides.

After the samples dried, I randomly designated half of the spots as control samples, while the other half were subjected to ozone treatment.

| Sample type | Experiment | Volume | Examined dilution | Indepen-dent repetition | # of samples / dilution |
|---|---|---|---|---|---|
| Blood | Luminol test | 10 µl | 1:1, 1:10, 1:100, 1:1000 | 6x | 6 |
| | Immunoassay | 10 µl | 1:1, 1:10, 1:100, 1:1000 | 3x | 6 |
| | RT-qPCR | 10 µl | 1:1 | 3x | 9 |
| | STR profiling | 1 µl | 1:1 | 3x | 3 |
| Saliva | Phadebas® test | 10 µl | 1:1 | 3x | 3 |
| | Immunoassay | 10 µl | 1:1, 1:10, 1:100, 1:1000 | 3x | 6 |
| | RT-qPCR | 10 µl | 1:1 | 3x | 9 |
| | STR profiling | 1 µl | 1:1 | 3x | 3 |
| Semen | Microscope | 10 µl | 1:1, 1:10, 1:100, 1:1000 | 3x | 6 |
| | STK Lab | 10 µl | 1:1, 1:10, 1:100, 1:1000 | 5x | 5 |
| | Immunoassay | 10 µl | 1:1, 1:10, 1:100, 1:1000 | 3x | 6 |
| | RT-qPCR | 10 µl | 1:1 | 3x | 9 |
| | STR profiling | 1 µl | 1:1 | 3x | 3 |

***Table 1.*** *Summary of the methods used and the examined dilutions for each type of body fluid. The number of samples indicates the total number of ozone-treated samples examined at each dilution, which is also equal to the number of corresponding control samples.*

### *Investigation of the effect of ozone treatment on DNA deposited during shedding*

To study the DNA deposited on objects that come into direct contact with the skin surface (shedding), I used surgical masks. Three volunteers wore sterile surgical masks for 2 hours. Afterward, for control samples, I collected a 1×1 cm section randomly from the nasal region and a 1 cm section from the elastic strap. After ozone treatment, I cut out the corresponding sections from the opposite region of the mask for further analysis.

#### Ozone treatment

For my experiments, I applied an ozone generator capable of producing 3500 mg/h of ozone (Vosoco Electronics Co.), placed in an airtight chamber with a volume of 100 liters.

During the experiments, the ozone concentration was monitored using an MQ131 sensor (Zhengzhou Winsen Electronics Technology Co., Ltd.). When the ozone concentration reached the 400-ppm range, I placed the samples on a sterile plate inside the chamber. After 20 minutes of ozone treatment, I turned off the generator and kept the

chamber sealed for 1 hour. Throughout the experiments, the internal temperature of the chamber ranged between 28-34°C, and the relative humidity was between 62-68%.

**Examination of blood samples with luminol**

During the luminol test, 10 ml of luminol stock solution (675 mM $Na_2CO_3$, 10 mM luminol) and 10 ml of 10% $H_2O_2$ were freshly mixed before each experiment.

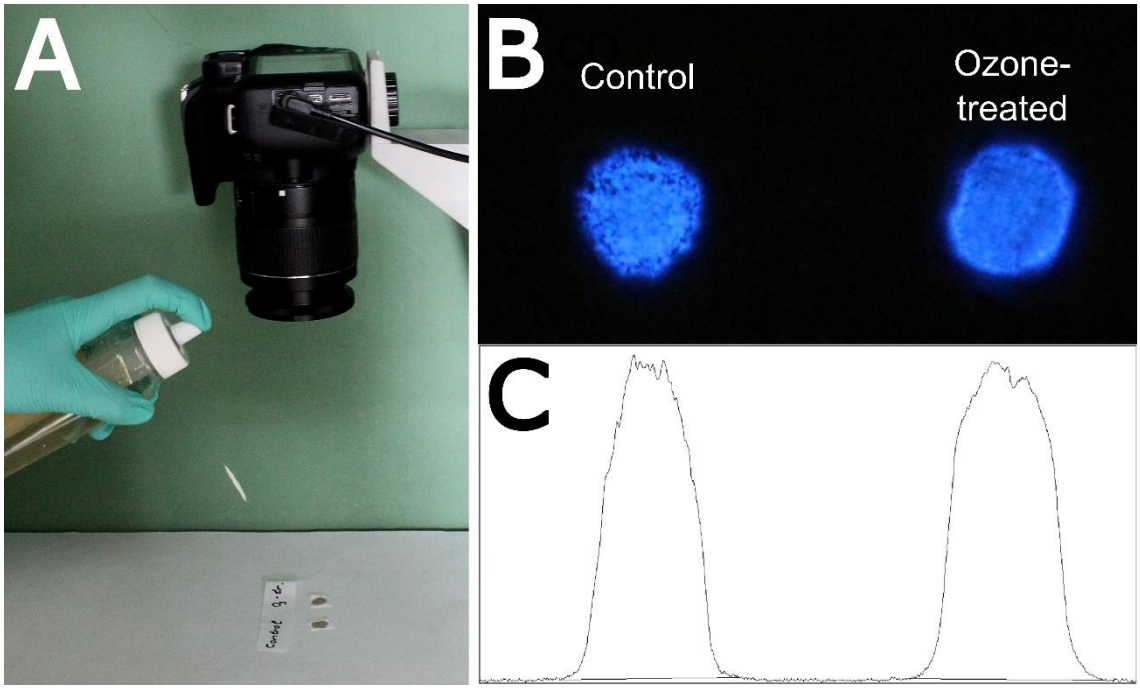


***Figure 1**. Investigation of the effect of ozone on luminol tests of blood samples.* ***A:** Parallel examination of a control and an ozone-treated blood sample. The experiment was conducted in complete darkness.* ***B:** Representative example of chemiluminescence occurring.* ***C:** Graphical representation of intensity using ImageJ software. The intensity values correspond to the area under the curve; for each pair of samples, the intensity value of the ozone-treated sample was compared to that of the control.*

The chemiluminescent light intensity may also be influenced by the amount of luminol in contact with the samples, so I worked with sample pairs for each examined dilution step to obtain the most consistent results possible. I placed a 10 µl control and ozone-treated blood sample side by side in a dark room, where I mounted a Canon 1300D digital camera on a vertical stand (**Figure 1.A**). I manually set the camera to the following

exposure values: shutter speed 1 s, aperture f: 9, ISO sensitivity 3200. I sprayed the luminol onto the sample pairs from the direction of the camera and recorded the resulting light reaction (**Figure 1.B**). Based on the images, I performed intensity calculations using ImageJ 1.53k software (**Figure 1.C**). For each pair of samples, I compared the intensity of the ozone-treated sample to that of the control and analyzed their ratio. The luminol experiment was repeated six times for each examined dilution step, starting from fresh blood samples each time.

**Investigation of α-amylase enzyme activity**

The activity of the α-amylase enzyme found in saliva was measured using the Phadebas® Amylase Test from 10 µl undiluted saliva spots. The blank sample for the test contained 4.2 ml of distilled water. For liquid control saliva samples, 10 µl of saliva was pipetted into 4.2 ml of distilled water. Samples spotted on fabric were cut out and placed in 4.2 ml of distilled water. I pre-incubated the samples at 37°C for 5 minutes, then added the provided test tablet to each tube with a 30-second delay. After thorough vortexing, the tubes were placed back in the water bath for 15 minutes. The reaction was precisely stopped at the end of 15 minutes by adding 1 ml of 0.5 M NaOH solution.

After centrifuging at 1500 g for 5 minutes, I pipetted 1 ml of the supernatant into a cuvette and measured the absorbance at 620 nm using an Implen NanoPhotometer

**Detection of semen stains with the STK Lab Kit**

During the STK Lab (STK® Sperm Tracker, AXO Science) screening test, I cut the filter paper provided by the manufacturer to the appropriate size. I placed the control sample and its ozone-treated pair side by side, then covered the semen stains with the filter paper moistened with distilled water and placed a weight on top. After 3 minutes, I illuminated the filter paper with a 365 nm UV lamp in a dark room. Photodocumentation was carried out similarly to the luminol test; a Canon 1300D digital camera was mounted on a vertical stand, and the exposure settings were manually adjusted as follows: shutter speed 1/160 s, aperture f: 9, ISO sensitivity 3200.

Fluorescent intensity values were calculated using the ImageJ 1.53k software, in the same manner as the evaluation of the luminol test. The experiment was repeated five times for each dilution step, starting with fresh semen samples each time.

**Microscopic examination of sperm**

For the microscopic examination of sperm, 10 µl of semen was pipetted directly onto slides for each dilution step. After allowing the samples to dry (~2 hours incubation at room temperature), one half of the slides served as the control group, while the other half underwent ozone treatment as previously described.

In each experiment, two control samples and two ozone-treated samples were processed for every dilution level. One control slide and one ozone-treated slide underwent traditional hematoxylin-eosin (H&E) staining, while their pairs were stained using the Christmas tree staining technique.

For the Christmas tree staining, I prepared the picro indigo carmine stain by dissolving 0.12 g of picric acid in 9 ml of distilled water with overnight stirring, followed by the addition of 0.003 g of indigo carmine, stirring until it was completely dissolved. During the staining process, the slides were incubated with Nuclear Fast Red (Thermo Fisher Scientific) for 15 minutes, which stains the head of the sperm. After washing with distilled water, a 15-second staining with picro indigo carmine followed. The slides were then washed with absolute ethanol and allowed to dry completely at room temperature.

The sperm were examined using a Nikon Alphaphot-2 YS2 microscope at 100x to 400x magnification. The entire experiment was repeated three times using fresh samples each time.

**Identification of tissue-specific antigens using immunochromatographic assays**

I used lateral flow immunoassays provided by the manufacturer SERATEC® for the detection of tissue-specific antigens. HemDirect, AmylaseTest, and PSA Semiquant kits were employed to identify human blood, saliva, and semen stains, respectively.

For hemoglobin detection, I added the cut-out samples to vials containing 1.5 ml of extraction buffer provided by the manufacturer. For the AmylaseTest, I pipetted 100 µl of extraction buffer into 1.5 ml centrifuge tubes, while for the PSA Semiquant, I used 500 µl of extraction buffer, and added the cut-out samples to these tubes. In all three tests, after incubating the samples at room temperature for 10 minutes with shaking, I pipetted 3 drops of the solution into the window of the test strip and recorded the results after 10 minutes.

The immunoassays were performed with duplicate samples at each dilution level and were repeated three times, each time starting with fresh samples, using separate dilution series and ozone treatments.

**Identification of tissue-specific mRNA markers**

RNA extraction from blood, saliva, and semen stains was performed using undiluted 10 µl sample volumes, immediately after the 1-hour waiting period following the ozone treatment. RNA extraction for both control and ozone-treated samples was carried out simultaneously using the RNeasy Plus Micro Kit (Qiagen), following the user-developed protocol "*Isolation of total RNA from ejectable buccal swabs*". The RNA samples were eluted in 14 µl of RNase-free distilled water.

| Sample type | Primer | Primer sequence | Amplicon length (bp) |
|---|---|---|---|
| blood | PF4 | AGGTCCGTCCCAGGCACATCACCAGC<br>CAGCGGGGCTTGCAGGTCCAAG | 125 |
| | HBB | CTGAGAACTTCAGGCTCCTGGG<br>CAGCAAGAAAGCGAGCTTAGTG | 159 |
| | HBB2 | AGGAGAAGTCTGCCGTTACTG<br>CCGAGCACTTTCTTGCCATGA | 190 |
| saliva | STATH | GGGTATGGCCCTTATCAGCC<br>CGTATTTGCCAATCAAGCCTCA | 135 |
| | HTN3 | CATGCTTTCCATGACTGGAG<br>AGTCCAAAGCGAATTTGCCA | 181 |
| semen | PRM1 | GCCAGGTACAGATGCTGTCGCAG<br>TTAGTGTCTTCTACATCGCGGTCT | 153 |
| | SEMG1 | CAACGACCGAAACCCATTA<br>TGTCATCCATGGACCAAGAA | 156 |
| house-keeping | 18S rRNA | CTCAACACGGGAAACCTCAC<br>CGCTCCACCAACTAAGAACG | 110 |
| | ACTB | TGACGTGGACATCCGCAAAG<br>CTGGAAGGTGGACAGCGAGG | 205 |

***Table 2.*** *Primer sequences of tissue-specific mRNA markers applied in the experiment.*

The synthesis of cDNA was performed using the Applied Biosystem High-Capacity cDNA Reverse Transcription Kit on a T100 Thermal Cycler (Bio-Rad).

The amplification of tissue-specific markers was carried out using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The primers used are detailed in **Table 2**.

The mRNA analysis was repeated three times, with each round involving 3 control and 3 ozone-treated samples per body fluid type.

**DNA extraction**

Before conducting the STR analysis, I examined the impact of ozone on isolated DNA. For this, control DNA was extracted from a fresh, sodium citrate-anticoagulated blood sample using the DNeasy Blood & Tissue Kit (Qiagen) following the "*Purification of total DNA from animal blood or cells*" protocol. In this experiment, I analyzed 9 control and 9 ozone-treated samples.

For other cases, such as re-extraction in control experiment, analysis of body fluids, and examination of DNA deposited on surgical masks, DNA extraction was performed using Chelex 100 beads (Bio-Rad). Samples were incubated with 200 µl of 10% Chelex solution at 56°C for 30 minutes and then at 100°C for exactly 8 minutes. The Chelex beads in the DNA solution were pelleted by a short 1-minute centrifugation at 14,000 rpm, and the supernatant containing the DNA was transferred to a new tube.

In the case of body fluids, undiluted 1 µl spots were examined in 3 independent repetitions. For the analysis of DNA deposited on surgical masks, a total of 9 masks were processed.

**DNA quantification, amplification, and separation**

DNA quantification was performed using the Qubit dsDNA HS Assay Kit following the manufacturer's instructions in all cases. DNA concentrations were measurable for samples from the control experiments and body fluids. However, for the DNA samples from the analysis of surgical masks, the concentrations were often below the sensitivity threshold. Therefore, for these samples, the amplification was conducted using the maximum amount of template DNA available.

The amplification of STR markers was carried out using the Investigator ESSplex SE QS Kit (Qiagen) according to the manufacturer's instructions. For the control experiments

and body fluid samples, 1 ng of DNA template was used. For the shedding study, the amplification was performed with the maximum possible amount of template DNA.

The separation of the amplicons was done using capillary gel electrophoresis on an ABI PRISM® 310 Genetic Analyzer (Thermo Fisher Scientific). Data analysis was carried out using the GeneMapper ID v3.2.1 (Applied Biosystems) software, applying a 50 RFU (Relative Fluorescence Unit) analytical threshold.

**Statistical analysis and diagrams**

The statistical analysis of both screening and confirmatory tests was performed using SPSS Statistics v28.0 software (IBM).

For evaluating the results of the luminol test and STK Lab kit, the Wilcoxon Signed-Rank test was employed to compare the intensity values between ozone-treated and control samples.

When analyzing the detection of tissue-specific RNA markers, we utilized the Kruskal-Wallis non-parametric one-way ANOVA for independent samples with multiple pairwise comparisons. This approach was used to determine differences between control and treated sample groups without assuming normal distribution.

In the assessment of STR profiles, we compared the number of detected alleles between the control and ozone-treated groups using SPSS Statistics v25 (IBM). A complete profile consisted of 34 detectable alleles.

When examining the effect of ozone on isolated DNA, the sample pairs, as well as the samples from the same mask were treated together. The Related Samples Wilcoxon Signed-Rank test was applied for this analysis. To compare the shedding capacities among different volunteers, the Kruskal-Wallis test was employed.

All diagrams were created using R software v4.2.0 (R Foundation, r-project.org), utilizing the ggplot2 library for data visualization.

# Results

## Examination of blood samples with luminol

The quantitative evaluation of the luminol test is challenging due to the transient nature of the chemiluminescent reaction, which typically lasts only 20-30 seconds and shows a decreasing intensity trend. To minimize discrepancies from capturing different moments of light intensity, both control and ozone-treated samples were evaluated in pairs to react simultaneously with luminol (**Figure 2.A**). Instead of comparing raw intensity values, the ratios of intensities between the ozone-treated and control samples were compared (**Figure 2.B**). For the calculations, the intensity value of the ozone-treated sample was divided by that of the control sample for each pair examined simultaneously.

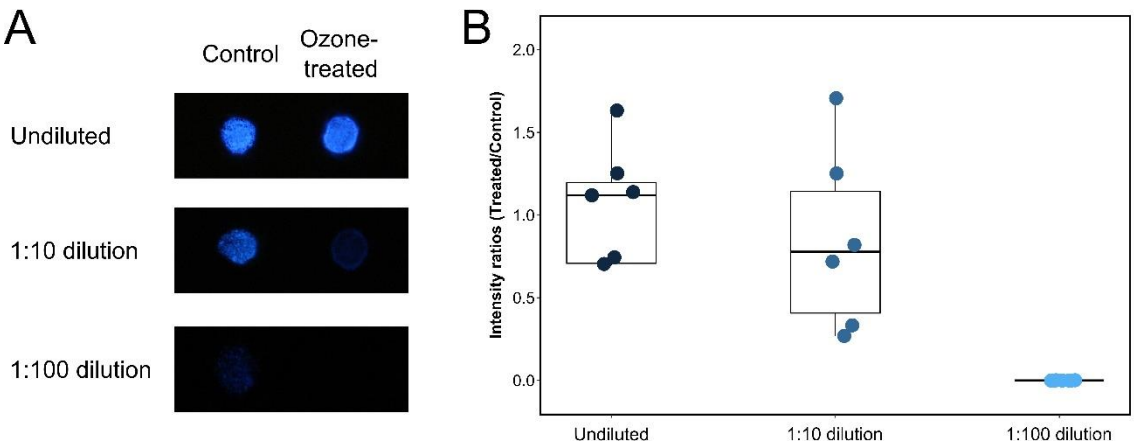


***Figure 2.*** *Effect of ozone treatment on the luminol test.* ***A:*** *Representative example of bloodstains' chemiluminescence. In each case, a control and an ozone-treated sample were simultaneously examined.* ***B:*** *The emitted light intensity was recorded using ImageJ software, and for each pair of samples, the intensity of the ozone-treated sample was divided by the intensity of the control.*

The average intensity ratio was close to 1 for both undiluted (mean, $\overline{x}$ = 1.04; SD = 0.33) and 1:10 diluted ($\overline{x}$ = 0.85; SD = 0.51) samples. The 1:100 diluted control samples showed very faint chemiluminescence, and no light reaction was detectable in the ozone-treated samples. At 1:1000 dilution, even the control samples did not exhibit chemiluminescence. There was no significant difference in the intensity values between the control and ozone-treated samples for the undiluted (z = -1.183, p = 0.237) and the

1:10 diluted (z = -1.153, p = 0.249) samples (Wilcoxon Signed-Rank Test). A significant difference was observed for the 1:100 diluted samples (z = -2.366, p = 0.018, r = 0.63), with a high effect size.

### Investigation of α-amylase enzyme activity

The amylase enzyme activity in saliva samples was determined based on absorbance values measured at 620 nm using a standard curve provided by the manufacturer. The absorbance of the blank sample was subtracted from the values of each sample in all cases.

The average absorbance of control samples was 1.04 (min = 0.942; max = 1.132; SD = 0.095), and all control samples exhibited high enzyme activity ($\overline{x}$ = 19,686 U/l; min = 18,680 U/l; max = 20,940 U/l; SD = 1150 U/l). In contrast, the absorbance and thus the enzyme activity of samples treated with ozone drastically decreased. In two samples, absorbance was not measurable, and in one sample, it was 0.203 ($\overline{x}$ = 0.067; min = 0.00; max = 0.203; SD = 0.117), indicating enzymatic activity degradation ($\overline{x}$ = 1720 U/l; min = 0 U/l; max = 5160 U/l; SD = 2979 U/l).

Due to the almost abolished enzyme activity in ozone-treated samples, diluted saliva samples were not examined using this method.

### Detection of semen stains with the STK Lab Kit

Similarly to the luminol test, I examined control-ozone-treated sample pairs in the case of the STK Lab kit simultaneously. The fluorescent intensity was recorded using ImageJ software, and by dividing the intensity of ozone-treated samples by that of controls, I obtained their ratio.

For undiluted samples, there was no difference in the fluorescent intensity ratio between treated and control samples ($\overline{x}$ = 1.016; SD = 0.113). However, at the 1:10 dilution, lower intensity was observed in ozone-treated samples (**Figure 3.A**), and the ratio decreased ($\overline{x}$ = 0.243; SD = 0.097) (**Figure 3.B**).

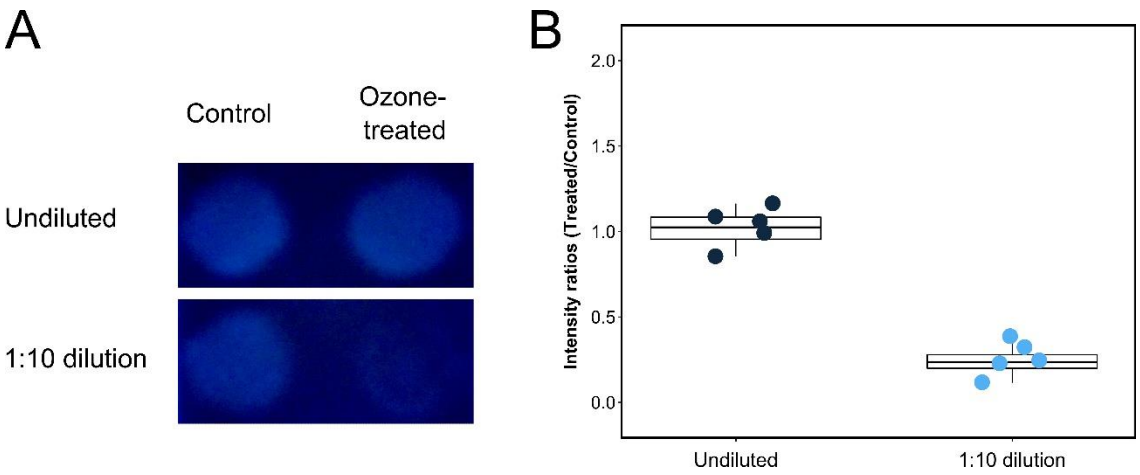


***Figure 3.*** *Detection of semen stains with STK Lab kit.* ***A:*** *Representative example of fluorescent signal from 10 µl semen stains.* ***B:*** *Ratio of intensity between ozone-treated and control sample pairs after measurement of fluorescent region intensity with ImageJ software.*

Statistically significant differences and a high effect size were observed when comparing the fluorescent intensity values of control and ozone-treated samples at the 1:10 dilution ($z = -2.023$, $p = 0.043$, $r = 0.64$), while there was no statistically significant difference for undiluted samples ($p = 1.000$). For the 1:100 and 1:1000 dilutions, neither control nor ozone-treated samples were detectable using this method.

**Microscopic examination of sperm**

During the light microscopic examination of native slides, no morphological differences were observed between the control and ozone-treated spermatozoa. Ozone treatment did not affect the staining procedures either; it did not cause any visible morphological changes. Therefore, both the H&E and Christmas tree staining methods effectively detected the head and tail parts of the spermatozoa.

**Identification of tissue-specific antigens using immunochromatographic assays**

Hemoglobin immunoassays conducted on 10 µl body fluid spots revealed that undiluted and 1:10 diluted ozone-treated samples showed detectable hemoglobin even after ozone treatment without a visible decrease in intensity on the test strip. Control samples remained positive even at a 1:1000 dilution, whereas ozone-treated samples failed to show hemoglobin presence at a 1:100 dilution.

In the case of seminal fluid, similarly to the hemoglobin test, the undiluted and 1:10 diluted ozone-treated samples showed positivity. Control samples exhibited detectable PSA presence at a 1:100 dilution, but ozone treatment showed negative results, parallel to the hemoglobin test. The 1:1000 dilution already fell below the detection limit of SERATEC® PSA Semiquant, so in this case I was not able to detect PSA in the control samples either.

In the α-amylase test, even the undiluted ozone-treated samples showed only very weak positivity, and in the case of the 1:10 diluted samples, the enzyme could only be detected in the control saliva samples. From a practical point of view, it is important to point out that even the 1:100 dilution fell below the detection threshold, potentially posing challenges if saliva becomes diluted with other body fluids.

**Identification of tissue-specific mRNA markers**

| | | $\Delta C_t$ ($O_3$-treated – control) | | Fold change ($O_3$-treated / control) | | | |
|---|---|---|---|---|---|---|---|
| | Primer | Mean | SD | Mean | SD | $C_t$ max | Efficiency (%) |
| blood | PF4 | 0,7 | 0,47 | 0,64 | 0,20 | 31,98 | 100 |
| | HBB | 0,9 | 0,52 | 0,56 | 0,21 | 21,34 | 100 |
| | HBB2 | 0,84 | 0,73 | 0,61 | 0,25 | 21,53 | 98 |
| | 18S rRNA | 1,37 | 1,02 | 0,46 | 0,36 | 19,47 | 100 |
| | ACTB | 2,29 | 0,41 | 0,22 | 0,06 | 29,68 | 94 |
| saliva | STATH | 2,75 | 0,32 | 0,15 | 0,04 | 39,12 | 100 |
| | HTN3 | 2,51 | 1,43 | 0,24 | 0,21 | 36,37 | 100 |
| | 18S rRNA | 1,05 | 0,78 | 0,53 | 0,26 | 20,25 | 100 |
| | ACTB | 2,03 | 0,69 | 0,27 | 0,13 | 36,09 | 99 |
| semen | PRM1 | 1,41 | 0,03 | 0,39 | 0,01 | 24,8 | 96 |
| | SEMG1 | 1,16 | 0,11 | 0,45 | 0,04 | 24,8 | 99 |
| | 18S rRNA | 0,65 | 0,47 | 0,68 | 0,20 | 11,29 | 90 |
| | ACTB | 0,88 | 0,08 | 0,55 | 0,03 | 26,95 | 100 |

***Table 3.*** *Comparison of Ct values of the tissue-specific mRNA markers.*

In the case of blood, 3 tissue-specific mRNA markers were detected alongside 2 housekeeping markers. For saliva and semen stains, 2 tissue-specific mRNA markers

were detected each, accompanied by 2 housekeeping markers. During the examination of primer pair efficiencies, 7 out of 13 cases exhibited 100% efficiency. The lowest efficiency, 90%, was observed for the 18S rRNA marker in semen sample.

Ozone treatment consistently caused mRNA degradation, indicated by increased threshold cycle ($C_t$) values compared to controls (**Table 3**). The largest increase was noted in saliva samples for the STATH marker, where the ozone-treated sample's Ct value increased by an average of 2.75 compared to the control, representing a 0.15-fold decrease in RNA quantity.

In semen samples, the 18S rRNA marker proved to be the most resistant to treatment, showing only a 0.65 $C_t$ value increase compared to the control. When comparing the fold changes in RNA quantity between sample types, a statistically significant difference was observed, accompanied by a high effect size ($H(2) = 7.792$, $p = 0.020$, $\eta^2 = 0.181$).

When examining the average quantitative changes of the three body fluid types, it became evident that the RNA content in saliva samples suffered significantly greater damage compared to blood ($p = 0.023$, $r = 0.385$) and semen ($p = 0.013$, $r = 0.422$). However, there was no statistically significant difference observed when comparing blood and semen samples ($p = 0.558$). The highest detected $C_t$ values were also associated with saliva samples, with three out of four markers showing $C_t$ values above 30 even in control samples. The highest $C_t$ value recorded was 39.12, observed in an ozone-treated saliva sample at the STATH marker.

**STR analysis**

In order to assess the applicability of ozone disinfection on sample types relevant to forensic genetics, I first examined its effect on isolated DNA to minimize factors influencing DNA integrity. Considering the initial DNA concentration (8.4 ng/µl) and the expected DNA quantity (0.84 ng/µl), it was determined that the re-extraction process was approximately 50% efficient even in control samples.

After examining 9 sample pairs, the DNA concentration in control samples ($n = 9$; $\overline{x} = 0.4$ ng/µl; $SD = 0.09$) was approximately three times higher than in ozone-treated samples ($n = 9$; $\overline{x} = 0.13$ ng/µl; $SD = 0.03$). There was a statistically significant difference observed between the medians ($p = 0.008$). Despite the concentration decrease, both control and ozone-treated samples yielded complete STR profiles.

Following ozone treatment, complete profiles were obtained from even from 1 µl undiluted blood, saliva, and semen stains, indicating that the ozone treatment did not hinder the profiling process.

During the analysis of DNA deposited during shedding, 9 surgical masks were processed, resulting in a total of 36 STR profiles being recorded. For each profile, autosomal alleles were counted in such way that the homozygotes were counted as two in the same way as the two heterozygous peaks within a locus (**Table 4**), resulting in a total of 34 alleles per complete profile.

| | EARLOOP | | NOSE | |
|---|---|---|---|---|
| | Control | $O_3$-treated | Control | $O_3$-treated |
| Contributor 1 | 34, 34 | 33, 31 | 30, 32 | 15, 28 |
| Contributor 2 | 34, 34, 32 | 27, 34, 34 | 15, 34, 34 | 22, 34, 34 |
| Contributor 3 | 33, 16, 18, 26 | 34, 17, 4, 29 | 7, 13, 30, 4 | 30, 11, 7, 8 |

***Table 4.*** *Number of detected alleles in profiles from masks of individual volunteers. The order of numbers corresponds to each mask.*

The median number of alleles in control samples was 31, complete profiles (34 alleles) were detected in 6 samples. In ozone-treated samples, 5 complete profiles were recorded, with a median allele number of 28.5 (**Figure 4.A**). There was no statistically significant difference ($p = 0.513$) observed in allele numbers between the control and ozone-treated sample groups upon comparison. During separate analyses of treatment and sampling locations (**Figure 4.B**), it emerged that while there was no significant difference in allele numbers between the control and ozone-treated sample groups, it is worthwhile to examine allele numbers from another perspective.

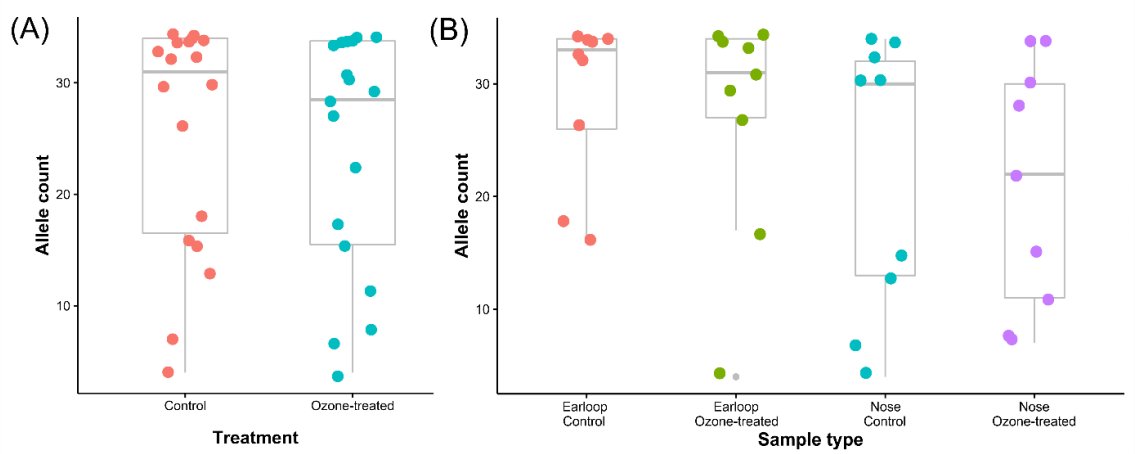


***Figure 4.*** *Detected allele numbers as a function of treatment and sampling location. Each point represents an individual sample.* ***A:*** *Comparison of control and ozone-treated samples irrespective of sampling location.* ***B:*** *Subgroup representation of allele numbers based on treatment and sampling location.*

Upon examining sampling locations, it became evident that the selection of the sampling site has a much greater impact on the success of profiling than ozone treatment itself. The average number of alleles in DNA samples from the earloops (i.e., aggregate of control and ozone-treated earloop samples) was 32.5, whereas it was only 25 for samples from the nasal area (**Figure 5**). A complete profile was recorded 7 times from earloop samples, compared to 4 times from nasal samples. When comparing the allele numbers from the two sampling sites, a statistically significant difference ($p = 0.011$) was observed.

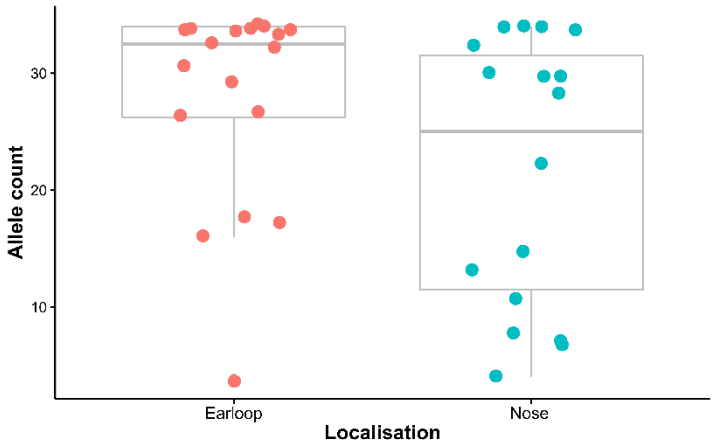


***Figure 5.*** *Allele numbers as a function of sampling location. A statistically significant difference ($p = 0.011$) was observed among allele numbers.*

Based on the allele numbers presented in **Table 4**, it is also noteworthy that the three volunteers exhibited different shedding capacities, indicating varying levels of ability to contaminate their surroundings with their DNA. Pairwise comparison of volunteers revealed a statistically significant difference (p = 0.001) between the second and third volunteers (**Figure 6**).

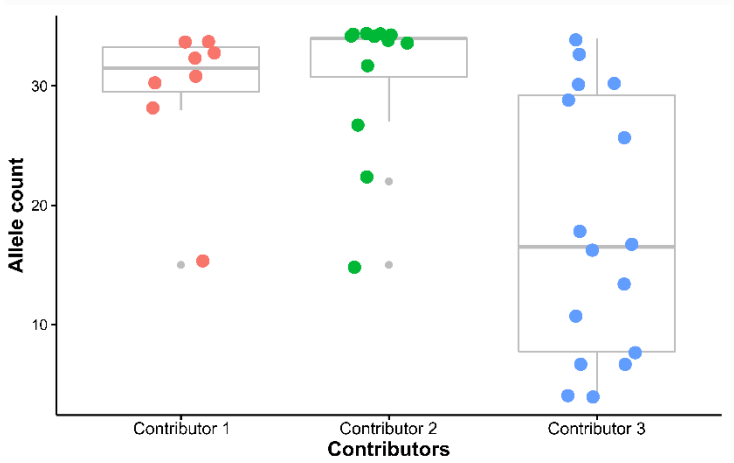


***Figure 6.*** *Comparison of allele numbers from samples collected from individual volunteers. A statistically significant difference (p = 0.001) was observed between volunteer 2 and volunteer 3.*

In addition to our findings suggesting that Contributor 3 is less prone to leaving behind genetic material, the experiment was based on the examination of surgical masks. Therefore, it is crucial not to overlook the significance of different physical characteristics such as head shape and size, as closer contact may result in more epithelial cells shedding from the skin surface.

## Discussion

The COVID-19 pandemic has highlighted situations in forensic genetics where there may be a need to disinfect samples, whether during another pandemic or in cases related to bioterrorism. Currently, there is no internationally accepted and established procedure for effectively inactivating pathogens from samples without potentially damaging biological material residues.

The application of ozone for disinfection has a long history, yet we still lack sufficient information regarding its application in this context. Due to its gaseous nature, ozone can

be applied easily and effectively even on porous surfaces, unlike liquid-based disinfection methods. Ozone can be readily generated from atmospheric oxygen, but it cannot be stored and must be produced on-site for each use.

In my experiments, I examined the effects of a 400-ppm ozone concentration on forensic investigations involving screening and confirmatory tests of blood, saliva, and semen stains. The body fluids were applied to white cotton fabric using a ten-fold dilution series. Screening tests included luminol, amylase, and acid phosphatase assays. As confirmatory tests, I performed immunochromatographic rapid tests and tissue-specific mRNA analysis for all three body fluids. For semen stains, I evaluated the detectability of spermatozoa microscopically using hematoxylin-eosin and Christmas tree staining techniques.

Prior to the forensic genetic analysis of body fluids, I investigated the effect of ozone disinfection on DNA solution from the point of view of STR profiling. Also, simulating a case that often occurs in forensics, I examined the effect of ozone treatment on inert surfaces directly exposed to DNA deposition, using surgical masks as test subjects. The results were consistently represented based on the number of alleles detected in the STR profiles.

During the luminol test for detecting blood, no differences were observed in chemiluminescent reactions between the undiluted and the 1:10 diluted samples. However, a significant difference was noted at a 1:100 dilution, where ozone-treated samples showed no chemiluminescence, indicating reduced sensitivity.

In the detection of amylase content in saliva stains, ozone-treated samples showed a substantial decrease in enzyme activity compared to control samples. Thus, ozone treatment hindered the identification procedure based on enzyme activity, likely due to oxidation of functional groups.

In contrast, during the detection of acid phosphatase from semen stains, positive results were obtained with undiluted and 1:10 diluted samples, but the 1:100 dilution fell below the detection limit of the STK Lab kit. Although the exact operating mechanism of the method employed is not fully understood, it has been demonstrated to be compatible for acid phosphatase detection post-ozone treatment, albeit with reduced fluorescence intensity in diluted semen samples.

In microscopic sperm identification using Christmas tree and hematoxylin-eosin staining, no changes in staining or morphological alterations were observed following ozone treatment.

When using immunochromatographic rapid tests for body fluid identification, reduced sensitivity was observed for all three body fluids. Specifically, in the amylase-specific test, only undiluted ozone-treated samples showed positivity, while in control samples, detection was successful up to a 1:10 dilution. In the PSA test, control samples were detectable up to a 1:100 dilution, whereas their ozone-treated counterparts showed positivity only up to a 1:10 dilution. Similarly, in the hemoglobin test, a pattern similar to PSA was observed, with the difference that even a 1:1000 dilution yielded positive results in control samples.

Identification of tissue-specific mRNA markers was successful for all three body fluids; however, degradation was observed compared to control samples. Specific to saliva markers, even control samples had high threshold values – close to the detection limit – and ozone treatment also resulted in the most significant damage observed in saliva samples.

When examining the effect of ozone treatment on pure DNA, although a decrease in the recovered DNA concentration was observed, full STR profiles were detectable in all cases, similar to the body fluid samples. Comparing profiles obtained from surgical masks revealed that the sampling location and individual characteristics played a more significant role in the success of obtaining an STR profile than the ozone treatment itself.

Throughout my research, I presented potential issues caused by ozone disinfection in forensic sample analysis. Generally, ozone treatment had some level of impact on almost every tested method, except for microscopic sperm analysis where no changes were observed. Ozone treatment most significantly hindered detectability in saliva samples. For blood and semen stains, no differences were observed in undiluted samples compared to controls, but sensitivity decreased in diluted samples. Although the ozone treatment did not generally lead to the failure in detecting specific nucleic acid sequences, it should not be ignored that if we are dealing with a forensic sample exposed to environmental influences, degraded, old, or containing a potentially low amount of DNA, in all cases the potential benefits and drawbacks of using ozone treatment should be determined with regard to the given risk of infection.

## Summary of new results

- Ozone treatment inhibited the presumptive testing of saliva stains (Phadebas® Amylase Test).
- Did not affect the identification of undiluted blood and semen stain with presumptive test (luminol and STK Lab, respectively).
- Sensitivity of luminol test decreased compared to the control.
- For the STK Lab kit, there was a decrease in signal intensity, but sensitivity remained unchanged in the examined range.
- Did not affect the microscopic identification of sperm cells.
- Immunochromatographic rapid tests showed reduced sensitivity.
- Did not hinder nucleic acid analysis.
- The saliva sample showed the highest $\Delta C_t$ value during the identification of tissue-specific mRNA markers.
- Caused decrease in the measurable concentration of DNA
- In the analysis of low-copy-number DNA samples, it was observed that individual characteristics and sampling site played a larger role in the success of STR profiling than ozone treatment.

## List of scientific publications and conference attendances

Total impact factor: 5.6
Total citations: 4
Independent citations: 3

**Scientific publications underlying the thesis:**

**V. Fejes**, D. Szucs, K. Sipos, V.S. Poor, Effect of ozone disinfection on forensic STR profiling, Forensic Sci. Int. 333 (2022) 111212. https://doi.org/10.1016/j.forsciint.2022.111212. IF: 2,2; Q1 D1

**V. Fejes**, G. Simon, L. Makszin, K. Sipos, V.S. Poor, Evaluation of the effect of ozone disinfection on forensic identification of blood, saliva, and semen stains, Sci. Justice 64 (2024) 151–158. https://doi.org/10.1016/j.scijus.2023.12.005. IF: 1,9; Q2

**Additional scientific publications:**

D. Szűcs, **V. Fejes**, Z. Kozma, V.S. Poór, K. Sipos, Sternal aspirate sampling of Bacillariophyceae (diatoms) and Cyanobacteria in suspected drowning cases, J. Forensic Leg. Med. 85 (2022). https://doi.org/10.1016/j.jflm.2021.102298. IF: 1,5; Q1

V.S. Poór, M. Alhabahbeh, **V. Fejes**, G. Kovács, Z. Pádár, DNA extraction and STR profiling from histological slides, Forensic Sci. Int. Genet. Suppl. Ser. 8 (2022) 176–177. https://doi.org/10.1016/j.fsigss.2022.10.026. IF: 0; Q3

Poór, V.S., **Fejes, V**., Szűcs, D., Simon, G, Postmortem Sampling of Cyanobacteria in the Case of Suspected Drowning. In: Thajuddin, N., Sankara narayanan, A., Dhanasekaran, D. (eds) Protocols for Cyanobacteria Sampling and Detection of Cyanotoxin. Springer, Singapore (2023) 189-194. https://doi.org/10.1007/978-981-99-4514-6_27

**Conference attendances:**

***Conference participations related to the thesis:***

Evaluation of the effect of ozone disinfection on forensic presumptive and confirmatory tests of blood, saliva, and semen stains

**Fejes Vivien**, Simon Gábor, Sipos Katalin, Poór Viktor Soma

2024.06.20-22. 29th International Meeting on Forensic Medicine Alpe-Adria-Pannonia

Effect of ozone disinfection on STR profiling

**Fejes Vivien**, Sipos Katalin, Poór Viktor Soma

2022.08.25-27. Magyar Igazságügyi Orvosok Társaságának XVII. Kongresszusa

Effect of ozone disinfection on forensic samples (poster)

**Fejes Vivien**, Szűcs Dominika, Sipos Katalin, Simon Gábor, Poór Viktor Soma

2022.06.22-27. 12th ISABS Conference (Dubrovnik, Horvátország)

Ozone disinfection of forensic samples

**Fejes Vivien**, Sipos Katalin

2022.04.30. Magyar Biotechnológus-hallgatók Egyesülete (Pécs)

***Conference participations not related to the thesis topic:***

Direct PCR from agarose and polyacrylamide gels

**Fejes Vivien**, Szűcs Dominika, Kozma Zsolt, Sipos Katalin, Poór Viktor Soma

2019.05.10-11. Fiatal Igazságügyi Orvosszakértők Fóruma (Debrecen)

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