Final Summary Overview

Preventing Hydrolytic and Oxidative Damage to Biological Evidence with Antioxidants and Chelators

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Purpose

In forensic science, proper evidence collection and storage techniques are important to prevent or minimize DNA degradation in biological samples. The principal processes involved in DNA degradation are hydrolysis and oxidation, which primarily occur when DNA is exposed to oxygen and water [1]. However, it may not always be possible to achieve ideal storage conditions for evidence, especially when stored for extensive periods of time. This presents a challenge in the forensic community where evidence may undergo long-term storage for months or years before it is processed. In addition, stored biological evidence has been increasingly reexamined in efforts to exonerate wrongfully convicted individuals and to solve cold cases. To preserve the integrity of the DNA and reduce the risk of DNA degradation, biological evidence should be preserved from environmental damage as soon as it is collected. The ability to apply a DNA preservative directly to the swab would minimize the risk of DNA degradation and could allow for the generation of higher quality DNA profiles. Currently, there are few options available to the forensic community for long-term preservation of biological evidence.

The goal of this research was to identify an effective method for long-term preservation of forensic DNA evidence using commercial-off-the-shelf (COTS) preservatives that can be applied directly to evidence collection substrates. To accomplish this goal, the preservative effects of three chelators and four antioxidants were examined on forensically relevant biological materials deposited on cotton swabs. Chelating agents and antioxidants interfere with DNA degradation caused by hydrolysis and oxidation. The chelating agents examined in this study were desferrioxamine (DFOA), diethylenetriamine pentaacetic acid (DTPA), and ethylenediaminetetraacetic acid (EDTA); and the antioxidants were α-tocopherol, astaxanthin,

hydroxytyrosol, and zinc. Each of the aforementioned preservatives have been shown to be effective chelators and antioxidants in other applications [2-8].

Methods

This study was divided into two distinct phases. In Phase I, α-tocopherol, astaxanthin, EDTA, DFOA, DTPA, hydroxytyrosol, and zinc were examined for long-term functionality on biological materials deposited on cotton swabs and stored for varying lengths of time under a range of environmental conditions. In Phase II, six application methods for applying preservatives to swabs were evaluated.

Phase I

Human blood and semen were purchased from Biological Specialty Corporation (Colmar, PA). Saliva, vaginal swabs, and buccal swabs were collected from internal donors under IRB guidelines with informed consent. Following collection, vaginal and buccal cells were eluted from their respective swabs into 1X phosphate buffered saline (PBS). No additional preservatives were added to the biological materials during or after collection. Blood (5 µl), semen (5 µl), saliva (35 µl), eluted vaginal cells suspended in 1X PBS (10 µl), and eluted buccal cells suspended in 1X PBS (10 µl) were applied to the tips of sterile cotton swabs (Puritan®, Guilford, Maine) (Table 1). The eluted buccal cell swabs were used to simulate mock touch evidence by preparing the swabs to contain approximately 0.5 ng DNA each. Additional blood samples were prepared by applying 5 µl blood to Whatman™ Non-Indicating FTA Classic Cards (GE Healthcare, Chicago, IL). These samples were included to determine if the COTS preservative treatments offer any advantages over FTA. Samples were prepared in triplicate for each biological sample type, time point, and preservative, with the exception of the mock touch samples, which were only prepared for the time

points indicated in Table 2. A total of 2,571 samples were prepared for this phase. Following application of the biological samples, all swabs were dried at room temperature for approximately one hour. The preservative solutions were prepared according to Table 3, and the solution formulations were selected based on a literature review. Using metered-dose dropper bottles, two drops (80 µl total) of chemical preservative were applied directly to the tip of each swab. After treatment with the preservatives, the swabs were dried at room temperature overnight, placed in cardboard swab boxes, and stored according to the conditions specified in Table 2. Sample storage occurred at room temperature, 37°C, and 50°C with ambient relative humidity conditions or 75-85% humidity. On average, the ambient relative humidity varied seasonally but did not exceed 60%, and the ambient relative humidity in the biological ovens was slightly below the humidity observed at room temperature. Increased humidity and temperature were used as environmental variables to accelerate the DNA degradation process [1], particularly in the blood and semen samples, which have been shown to be relatively stable over time under ambient humidity conditions [9]. Increased storage temperature was also used to perform accelerated sample aging when paired with ambient humidity.

Due to the time constraints of the study, sample aging was performed using two methods: real-time aging by storing samples at room temperature and accelerated aging by storing samples at either 37°C or 50°C. Accelerated aging is a technique used to simulate aging of medical devices when real-time aging is not feasible, and it has previously been used to simulate the aging of DNA extracts [10]. The simplified protocol for accelerated aging, or the 10-degree rule, was utilized for this study [11,12]. This protocol states that a temperature increase of 10°C corresponds to a twofold increase in shelf life ($Q_{10} = 2$):

$$\mathbf{TIME_{T1} = TIME_{RT}/Q_{10}^{(T_1 - T_{RT})/10}}$$

where T1 = oven aging temperature, T_{RT} = room temperature (22°C), and Q_{10} = reaction-rate coefficient [11]. The real-time room temperature aging (RT) and accelerated aging (AA) studies were conducted simultaneously. For each biological sample type, samples were tested at the time points specified in Table 2.

Samples were extracted on the Qiagen® QIAsymphony® robot with the QIAsymphony DNA Investigator® Kit. After DNA extraction, 2 µl of each sample was quantified using the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher, Waltham, MA) with 11 µl reaction volumes on the Applied Biosystems® 7500 Real-Time PCR System. Samples that contained less than 0.067 ng/µl of DNA were concentrated with Microcon DNA Fast Flow columns (MilliporeSigma, Burlington, MA). A template DNA quantity of 0.5 ng was targeted for amplification. When a sample contained less than 0.5 ng DNA, the entire sample was added to the amplification reaction. Amplification was performed with the GlobalFiler™ PCR Amplification Kit (Thermo Fisher, Waltham, MA) using 12.5 µl reaction volumes and 28 amplification cycles. Capillary electrophoresis was performed on the 3500xL Genetic Analyzer for Human Identification (Thermo Fisher, Waltham, MA), and data was analyzed with GeneMapper™ ID-X v1.5 using an analytical threshold of 125 RFU and a stochastic threshold of 600 RFU.

Quantifiler Trio Degradation Index (DI) values were evaluated for each sample. DI values <1 indicated that the DNA was not degraded. DI values 1-10 indicated that the DNA was slightly to moderately degraded. DI values >10 indicated that the DNA was significantly degraded. Additional data evaluations were performed by assigning a quality score between 0.05 and 10 to each profile based on the forensic DNA profile index (*FI*) developed by Hedman et al [13]. This index assesses the quality of a DNA profile by providing a single quantitative value that takes three factors into consideration: overall peak height, peak height balance within each locus in a profile,

and peak height balance across all loci in a profile. A score of 10 represented the highest quality profiles. JMP® Statistical Discovery™ Software v12.2 (SAS® Institute Inc., Cary, NC) was used to perform all statistical analyses. Tukey's HSD tests, Dunnett's test, f-tests for variance, and two-tailed t-tests were employed to perform comparisons as appropriate. Comparisons between time points within a preservative treatment were performed using Dunnett's test with the 0-day samples as the control.

Phase II

In Phase II, six methods for applying preservative solutions to a collection swab were evaluated. Three methods involved using metered-dose pharmaceutical liquid dispensers including a dropper bottle, nasal spray bottle, and oral spray bottle. These applicators are designed to dispense the same amount of liquid with each use (40 µl of liquid for the dropper bottle, and 120 µl for the oral and nasal spray bottles). Atomizers, unmetered applicators that dispense liquid by creating a fine mist of droplets after compressing the nozzle of the device, were also examined. The last two preservative application methods tested in this study involved soaking clean swabs directly in a preservative and allowing them to either dry as a preservative pretreatment or remain wet for use as the moistening agent when swabbing.

The six applications methods were evaluated to determine their ease of use and learning. Ten volunteers unfamiliar with the methods were provided written instructions and a verbal overview of each application method. Volunteers then performed each method on mock sample swabs and immediately provided user feedback using a standardized questionnaire, which employed seven point Likert scales to allow volunteers to rate their agreement with statements regarding the methods from (1) strongly disagree to (7) strongly agree. Median Likert score values

were determined for each method and questionnaire statement, and the Mann-Whitney U Test was used to determine if the Likert scores were significantly different between the application methods.

Additionally, an evaluation was performed to determine whether the preservatives retain their efficacy over time when deposited on swabs with the various application methods. To accomplish this, each application method was used to deposit preservative solutions on saliva swabs prepared according to the methods detailed for Phase I. For the pretreated and moistened swab application methods, the preservative solutions were applied to the swabs prior to application of saliva. Due to time constraints, EDTA, hydroxytyrosol, and zinc were selected for this phase based on the preliminary results they produced as of the Phase I 183-day time point. Three preservatives were examined to maximize the likelihood that effective preservation would be observed. For each application method and time point, samples and untreated controls were prepared in triplicate. A total of 399 samples were prepared for Phase II and stored according to the conditions specified in Table 4. Sample processing and data analysis were performed according to the methods detailed for Phase I.

Findings

Phase I

Phase I findings are reported in two manuscripts [14,15], which are under peer review. Here, the key findings are summarized. Until the manuscripts are accepted for publication, further detail can be obtained from the authors.

Finding 1: In the first paper under review, Bathrick and Davoren examine the effectiveness of the accelerated aging technique for evidence-type biological samples [14]. Statistical analysis of the DI and *FI* values demonstrated that the samples stored under real-time aging conditions and their

accelerated aging counterparts were equivalent (Figure 1) and that accelerated aging at 37˚C and 50˚C for an equivalent of up to 548 days is a viable alternative for real-time aging of blood, saliva, semen, and vaginal cell samples. When using accelerated aging for forensic studies, we recommend following the procedures used in medical device manufacturing [16]: conduct real-time aging studies in parallel to accelerated studies, consider expiry dates established using accelerated aging data tentative until real-time data are available, and note that data obtained are based on conditions simulating the effects of aging on the materials.

Finding 2: In the second paper under review, Bathrick and Davoren discuss the efficacy of the examined preservative solutions [15]. In general, *FI* values decreased over time for each biological material and preservative (Figure 2). No statistically significant differences in STR profile quality were reliably observed between the treated and untreated blood and semen samples. Following an evaluation of the profile quality trends exhibited by the preservative-treated saliva, vaginal cell, and mock touch samples over time, EDTA was found to the only preservative solution that produced higher *FI* values than the untreated samples, which indicates higher quality STR profiles. Statistically significant increases in *FI* were observed for saliva at the 1550, 1274, 2542, and 3816-day AA time points (all $p < 0.001$), vaginal cells at the 1274, 2542, and 3816-day AA time points ($p = 0.009$, $p < 0.001$, $p < 0.001$), and mock touch at the 1032 and 2542-day AA time points ($p = 0.001$, $p < 0.001$).

Finding 3: In the second paper under review, Bathrick and Davoren discuss an interaction observed between increased humidity and EDTA treatment [15]. Increased humidity was utilized to promote degradation of the biological materials; however, elevated humidity was found to have an adverse effect on EDTA treatment and resulted in significantly reduced STR profile quality when

compared to the untreated samples (Figure 3). A similar effect was observed for EDTA-treated samples at various RT time points with ambient humidity when compared to the untreated samples. Although mock touch samples were not examined with 75-85% humidity, the samples stored at 365 days RT exhibited a similar trend as the other RT biological materials. Overall, these results indicated that, when performing similar studies, humidity is an important environmental variable to investigate to ascertain whether there are any unanticipated interactions between the humidity, biological material(s), and preservative(s).

Finding 4: In the second paper under review, Bathrick and Davoren discuss the stability of the untreated biological materials [15]. The untreated saliva, vaginal cell, and mock touch samples appeared to be inherently prone to degradation as demonstrated by a general decline in profile quality over time, whereas untreated blood and semen were shown to be the most stable biological sample types, with fairly consistent profile quality results obtained across all time points (Figure 2). Based on these observations, future or similar studies may focus on minimizing DNA degradation in saliva, vaginal cell, and mock touch samples.

Phase II

Finding 1: The user evaluations and preservative efficacy results were both considered when evaluating the preservative application methods [17]. Data from the hydroxytyrosol- and zinc-treated samples are not presented as they were demonstrated to be ineffective in Phase I after processing the post-183-day time points. When evaluated for ease of use and learnability, the nasal and oral spray methods produced the lowest median Likert scores (Table 5), which were significantly lower for many statements pertaining to ease of use when compared to the other application methods (Table 6). Additionally, the nasal and oral spray bottles were the only methods

that produced significantly lower Likert scores for statements pertaining to ease of learning when compared to the other application methods. During the preservative efficacy evaluation, statistically significant differences in both mean DI and corresponding *FI* were not observed when comparing results from the nasal and oral spray methods to the other preservative methods. Based on this and their poor performance during the user evaluation, the nasal and oral spray methods were eliminated from further consideration. The mean DI and *FI* data from these methods were included during statistical analysis with Tukey HSD but are not presented as these methods are not considered viable options based on the user evaluations.

When evaluated for ease of use and learnability, the pretreated swab method produced the highest median Likert scores (Table 5); however, the metered dropper bottle, atomizer, and moistened swab methods each only differed significantly from the pretreated swabs at one ease of use statement (Table 6). Regardless, it is the opinion of the researchers that the atomizer method is not a viable option for use in the field or laboratory. When preparing Phase II samples, the researchers observed that the atomizer method dispersed a substantial amount of preservative solution into the air and surrounding area when used on the swabs, which is not desirable for samples prepared outside of a hood. To evaluate preservative efficacy, mean DI and *FI* values were determined (Figure 4) and compared (Table 7) for each application method at each time point. The dropper bottle, atomizer, pretreated swab, and moistened swab preservative application methods resulted in mean DI values that were significantly lower than the untreated samples at the later time points (Table 7). Some statistically significant differences in DI values were observed when the aforementioned methods were compared to each other (Table 7), and the practical implications of these differences on STR profile quality were observed by examining the corresponding *FI* values (Figure 4). Variations in DI were only considered impactful when a

statistically significant difference in corresponding *FI* was also observed (Table 7). The dropper bottle method was the only application method at any time point that resulted in significant differences in both DI and *FI* when compared to other application methods. The dropper bottle method also produced mean DI values less than or approximately equal to 1 at every time point except the 183- and 365-day RT time points, which demonstrated increased mean DI values and decreased mean *FI* values. A similar effect in the EDTA-treated saliva and vaginal cell samples was discussed in Phase I Finding 3, which indicates the issue is related to the EDTA solution rather than the various application methods.

Based on the user evaluations, researcher observations, and preservative efficacy results, the metered dropper bottle, pretreated swab, and moistened swab methods are effective and practical for the application of preservative solutions to swabs.

Implications for Criminal Justice Policy and Practice

This study examined methods for preserving biological materials collected on swabs and stored for extensive periods of time. During criminal investigations, evidence samples are frequently collected on sterile cotton tipped swabs and can be stored for months or years while awaiting state or federal funding for laboratory processing. Biological evidence can also be stored for long periods of time after the initial processing has been completed. In that time, DNA evidence may become degraded due to hydrolysis or oxidation. Currently, few procedures are in place to prevent sample degradation prior to DNA extraction. Application of a preservative could help protect the quality of biological evidence samples, regardless of when they are processed. Antioxidant and chelator solutions may have direct applications for the preservation of biological forensic evidence. These COTS preservatives are inexpensive, safe, simple to prepare, and could easily be applied to the cotton swab by forensic investigators at crime scenes. This study presents

a novel method for preservation of evidence samples collected on swabs. Application of EDTA solution to cotton swabs using metered dropper bottle bottles, pretreated swabs, or moistened swabs does not require expensive instruments or specialized skills and can easily be adopted by any crime scene unit/investigator or crime laboratory regardless of funding level. Preserving biological evidence for potential long-term storage may aid in not only the prosecution of cold cases but also the exoneration of the innocent as DNA technology advances.

Scholarly Products

The findings for this study are reported in two manuscripts, which have been submitted to scholarly publications and are under peer review. Dissemination of data also has occurred or is anticipated to occur through two or more poster presentations (in-person or virtual).

1. Bathrick AS, Davoren JD. Evaluation of Methods for the Application of Preservative Solutions to Saliva on Swabs. Poster made available online for Bode 2020 Virtual Forensic DNA Conference. October 26-27, 2020. https://bit.ly/2VBOA8K
2. Bathrick AS, Davoren JD. Accelerated Aging of Forensically Relevant Biological Materials on Swabs. Under review 2020.
3. Bathrick AS, Davoren JD. Preservation of Forensically Relevant Biological Materials with Commercial Off-the-Shelf Antioxidants and Chelators. Under review 2020.
4. Bathrick AS, Davoren JD. Preservation of Forensically Relevant Biological Materials with Commercial Off-the-Shelf Antioxidants and Chelators. Poster submitted to 2021 NIJ Forensic Science R&D Symposium. Virtual. Anticipated presentation date February 16, 2021.

Tables and Figures

Table 1: Phase I sample types

Sample Type	Treatment							
	None	α-tocopherol	Astaxanthin	DFOA	DTPA	EDTA	Hydroxytyrosol	Zinc
Blood	75 swabs 75 FTA	75	75	75	75	75	75	75
Semen	75	75	75	75	75	75	75	75
Saliva	75	75	75	75	75	75	75	75
Vaginal Fluid	75	75	75	75	75	75	75	75
Mock Touch	12	12	12	12	12	12	12	12

Table 2: Storage time and conditions for Phase I blood (B), mock touch (MT), saliva (SA), semen (SE), and vaginal cell (VC) samples

Temperature	Relative Humidity	Time Stored	Function of Temperature	Accelerated Aging Equivalent (Days)	Sample Type	Total Samples
Room Temp	Ambient	0 days	N/A	N/A	B, MT, SA, SE, VC	123
Room Temp	Ambient	183 days	N/A	N/A	B, SA, SE, VC	99
		365 days	N/A	N/A	B, MT, SA, SE, VC	123
		548 days	N/A	N/A	B, SA, SE, VC	99
	75-85%	183 days	N/A	N/A	B, SA, SE, VC	99
		365 days	N/A	N/A	B, SA, SE, VC	99
		548 days	N/A	N/A	B, SA, SE, VC	99
37°C	Ambient	65 days	Accelerated Aging	183	B, SA, SE, VC	99
		129 days	Accelerated Aging	365	B, SA, SE, VC	99
		183 days	Accelerated Aging	518	B, SA, SE, VC	99
		194 days	Accelerated Aging	548	B, SA, SE, VC	99
		365 days	Accelerated Aging	1032	B, MT, SA, SE, VC	123
		548 days	Accelerated Aging	1550	B, SA, SE, VC	99
	75-85%	183 days	Environmental Variable	N/A	B, SA, SE, VC	99
		365 days	Environmental Variable	N/A	B, SA, SE, VC	99
		548 days	Environmental Variable	N/A	B, SA, SE, VC	99
50°C	Ambient	26 days	Accelerated Aging	183	B, SA, SE, VC	99
		52 days	Accelerated Aging	365	B, SA, SE, VC	99
		79 days	Accelerated Aging	548	B, SA, SE, VC	99
		183 days	Accelerated Aging	1274	B, SA, SE, VC	99
		365 days	Accelerated Aging	2542	B, MT, SA, SE, VC	123
		548 days	Accelerated Aging	3816	B, SA, SE, VC	99
	75-85%	183 days	Environmental Variable	N/A	B, SA, SE, VC	99
		365 days	Environmental Variable	N/A	B, SA, SE, VC	99
		548 days	Environmental Variable	N/A	B, SA, SE, VC	99

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Table 3: Preservative preparation protocols

Preservative	Protocol	Citation
α-Tocopherol	A 50 µg/mL α-tocopherol solution was prepared in ethanol.	2
Astaxanthin	A 5 µM astaxanthin solution was prepared in ethanol.	3
DFOA	A 1 mM DFOA solution was prepared in sterile water.	4
DTPA	A solution of 0.005 M DTPA and 0.01 M calcium chloride was prepared in sterile water. The solution was buffered to pH 7.3 with 1 M triethanolamine and HCl.	5
EDTA	A 0.2 M EDTA solution was prepared in sterile water.	6
Hydroxytyrosol	A 10 mM hydroxytyrosol stock solution was prepared in ethanol. The stock solution was diluted to 800 µM in sterile water prior to use.	7
Zinc	A 10X stock solution of 5% w/v zinc chloride, 5% w/v zinc trifluoroacetate, and 0.5% w/v calcium acetate was prepared in 1 M Tris-HCl, pH 7.4. The solution was diluted to 1X in sterile water prior to use.	8

Table 4: Storage conditions and time points for Phase II samples.

Temperature	Relative Humidity	Time Point	Function of Temperature	Accelerated Aging Equivalent (Days)	Samples
Room Temp	Ambient	0 days	N/A	N/A	57
Room Temp	Ambient	183 days	N/A	N/A	57
		365 days	N/A	N/A	57
37°C	Ambient	183 days	Accelerated Aging	518	57
		365 days	Accelerated Aging	1032	57
50°C	Ambient	183 days	Accelerated Aging	1274	57
		365 days	Accelerated Aging	2542	57

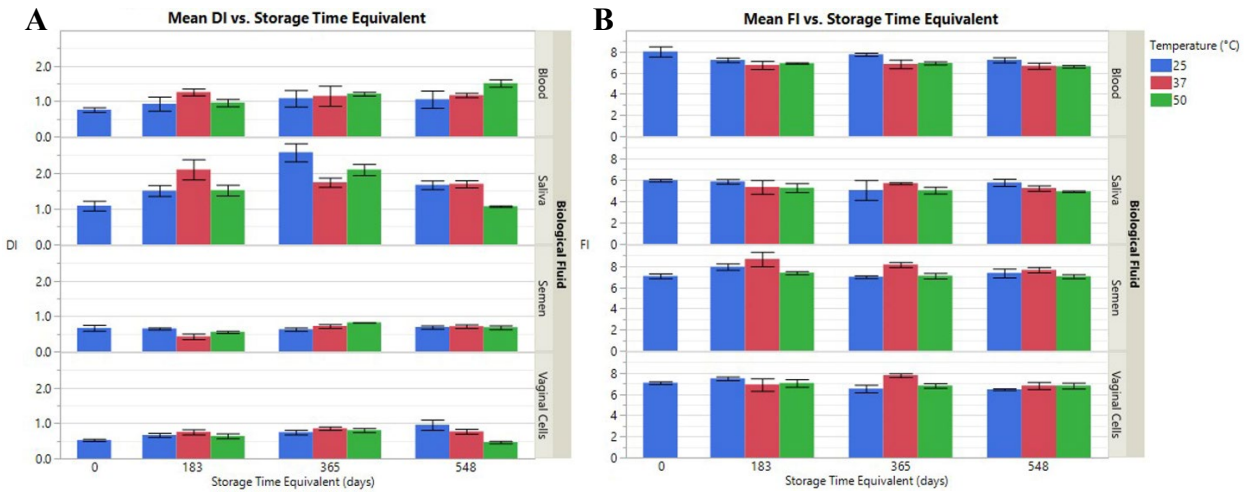

Figure 1: For the accelerated aging evaluation, mean (A) DI and (B) *FI* values for each biological material, storage temperature, and storage time equivalent were compared. DI values <1 indicate that the DNA was not degraded. DI values 1-10 indicate that the DNA was slightly to moderately degraded. An *FI* of 10 represents the highest quality profiles. Each error bar is constructed using one standard deviation from the mean.

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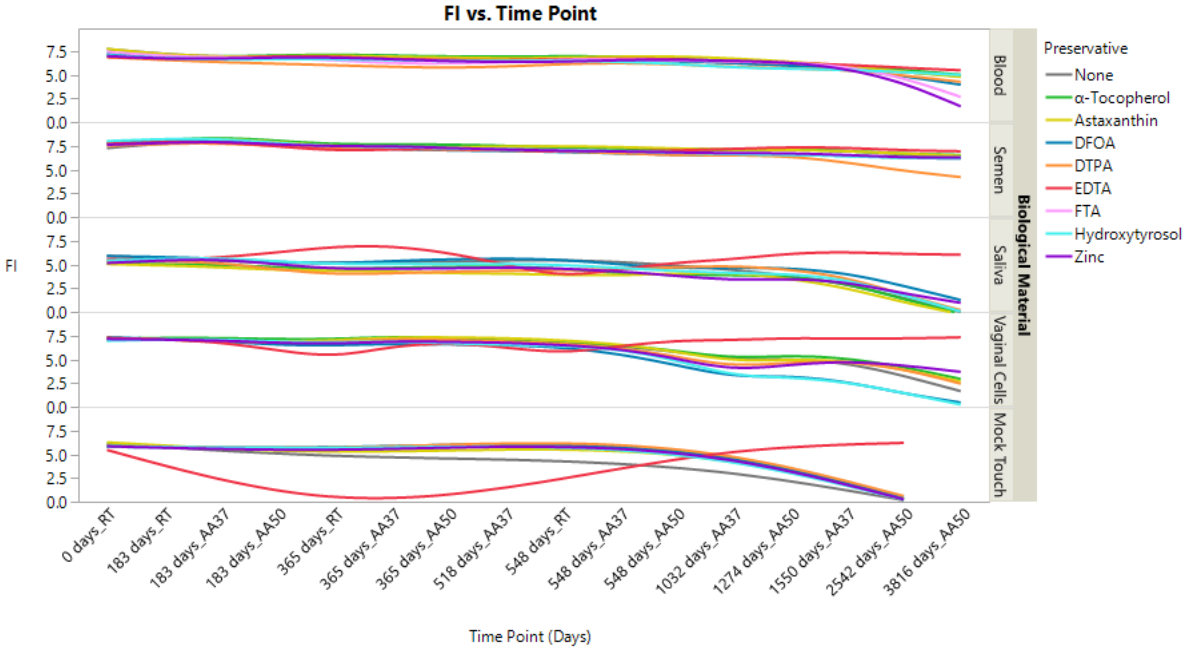

Figure 2: Mean *FI* values obtained for biological samples that were treated with α-tocopherol, astaxanthin, DFOA, DTPA, EDTA, FTA, hydroxytyrosol, and zinc were plotted for each storage time equivalent. The untreated control samples are represented as "None." Mock touch samples were only tested at the 0-day, 365-day RT, 1032-day AA, and 2542-day AA time points. In general, mean *FI* values decreased over time for each biological material and preservative. The EDTA-treated mock touch, saliva, and vaginal cell samples generated significantly higher *FI* values when compared to the untreated controls at several time points.

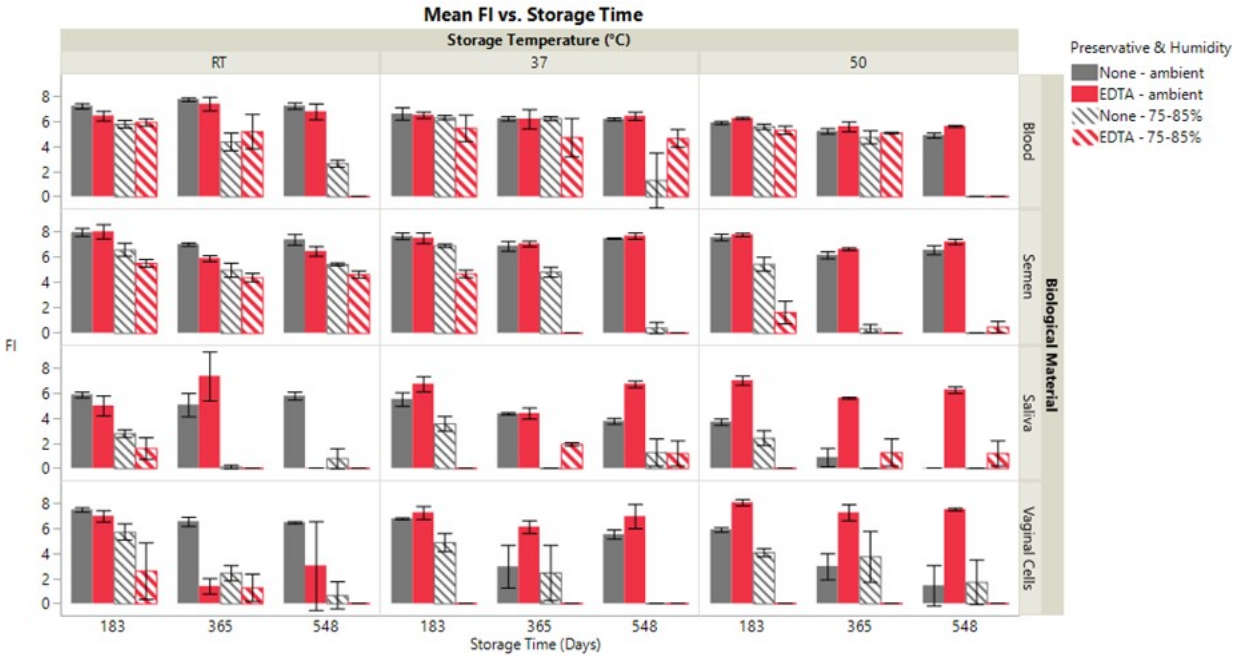

Figure 3: Mean *FI* for untreated and EDTA-treated samples stored at ambient and 75-85% humidity were compared for each storage temperature, storage time, and biological material. Each error bar is constructed using one standard deviation from the mean.

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Table 5: Median Likert scores obtained for each application method and questionnaire statement: (1) Strongly Disagree, (2) Disagree, (3) Somewhat Disagree, (4) Neutral, (5) Somewhat Agree, (6) Agree, and (7) Strongly Agree

Questionnaire Statement	Atomizer	Dropper Bottle	Oral Spray	Nasal Spray	Moistened Swab	Pretreated Swab
The application device/ method is easy to use.	7	6.5	5	5	7	7
The application device/ method is simple to use.	7	7	5	5	7	7
It is user friendly.	7	6	4	3.5	7	7
The application device/ method requires the fewest steps possible to apply the preservative.	6	3.5	5	5	7	7
Using the application device/ method is effortless.	6.5	5.5	4.5	3.5	6.5	7
The application device is easy to pump/ dispense.	7	5.5	4	2	6	7
It is easy to administer the preservative from the application device.	7	6.5	3.5	4	6	7
The application device is comfortable to hold when applying the device.	7	6.5	4	4	6	6.5
The application device fits comfortably in the hand when applying the preservative.	7	7	4.5	4	6	6.5
I can use the application device/method without written instructions.	6.5	7	3.5	5.5	7	7
I don't notice any inconsistencies as I use it.	5	2.5	4	2	4	6
I liked using the application device/ method.	7	3.5	2	3.5	6	6.5
I learned to use it quickly.	7	7	5	6.5	7	7
I easily remember how to use it.	7	7	6.5	6.5	7	7
It is easy to learn to use it.	7	7	5.5	6.5	7	7
I quickly became skillful with it.	7	6.5	5.5	5.5	7	7
Total	108	94.5	71.5	72	103.5	109.5

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Table 6: U values generated after comparing Likert scores with a two-tailed Mann-Whitney U test, $p < 0.05$, $n_1=n_2=10$, critical value = 23

Application Methods Compared†	The application method is easy to use.	The application method is simple to use.	It is user friendly.	The application method requires the fewest steps possible to apply the preservative.	Using the application method is effortless.	The application device is easy to pump/dispense.	It is easy to administer the preservative from the application device.	The application device is comfortable to hold when applying the preservative.	The application device fits comfortably in the hand when applying the preservative.	I can use the application method without written instructions.	I don't notice any inconsistencies as I use it.	I liked using the application method.	I learned to use it quickly.	I easily remember how to use it.	It is easy to learn to use it.	I quickly became skillful with it.
Atomizer Dropper Bottle	35	39.5	36	38.5	37.5	26.5	35	33	45.5	39.5	28	20*	40	35	44.5	41
Atomizer Oral Spray	6.5*	13*	5.5*	32	5*	2.5*	9.5*	9*	17.5*	14*	25.5	7.5*	10*	25	17.5*	29
Atomizer Nasal Spray	12*	16*	10.5*	35	12*	3.5*	10*	14*	15.5*	35	19.5*	19.5*	25	25	28.5	28
Dropper Bottle Oral Spray	32.5	24.5	27.5	47.5	38.5	18*	26.5	22.5*	26	13.5*	47	32	14*	35.5	22.5*	44
Dropper Bottle Nasal Spray	28.5	24	26.5	49.5	30	16.5*	25	26	23.5	33	44	42.5	33	38.5	34	43.5
Dropper Bottle Moistened Swab	40	46	37	24	37.5	43.5	50	48	43	48.5	47.5	29.5	45	40	49	42
Oral Spray Nasal Spray	45.5	41.5	46	49.5	39.5	38	46.5	48	43	22.5*	46	42	30	45.5	37	47
Moistened Swab Atomizer	42.5	42	48	11*	47.5	28.5	31	31.5	34.5	41	28.5	36	45	45	45	48
Moistened Swab Oral Spray	14.5*	13*	7*	4*	7.5*	11.5*	22*	14.5*	26	11*	47.5	14*	12*	28.5	20*	31
Moistened Swab Nasal Spray	18.5*	18*	12*	9*	13*	13*	22.5*	21.5*	22.5*	30	41	27	29	29.5	32	29.5
Pretreated Swab Atomizer	39.5	44.5	41	11*	42	44.5	46	36.5	41	48	39	48	45	45	50	38
Pretreated Swab Dropper Bottle	28	34	31	24	32.5	30.5	33	43.5	47.5	43	24	20.5*	46	40	44.5	32
Pretreated Swab Oral Spray	1*	7*	3.5*	4*	5*	4.5*	10*	12*	21.5*	14*	16*	7.5*	15.5*	28.5	17.5*	20*
Pretreated Swab Nasal Spray	6.5*	12*	8.5*	9*	10*	6*	10.5*	18.5*	18.5*	35	15.5*	19.5*	31	29.5	28.5	20*
Pretreated Swab Moistened Swab	33.5	35	40	50	40.5	34	29.5	45	42.5	44	22*	37.5	49.5	50	45	37

† When significant differences were detected, the application method listed first for each comparison received higher Likert scores.
* $U < 23$

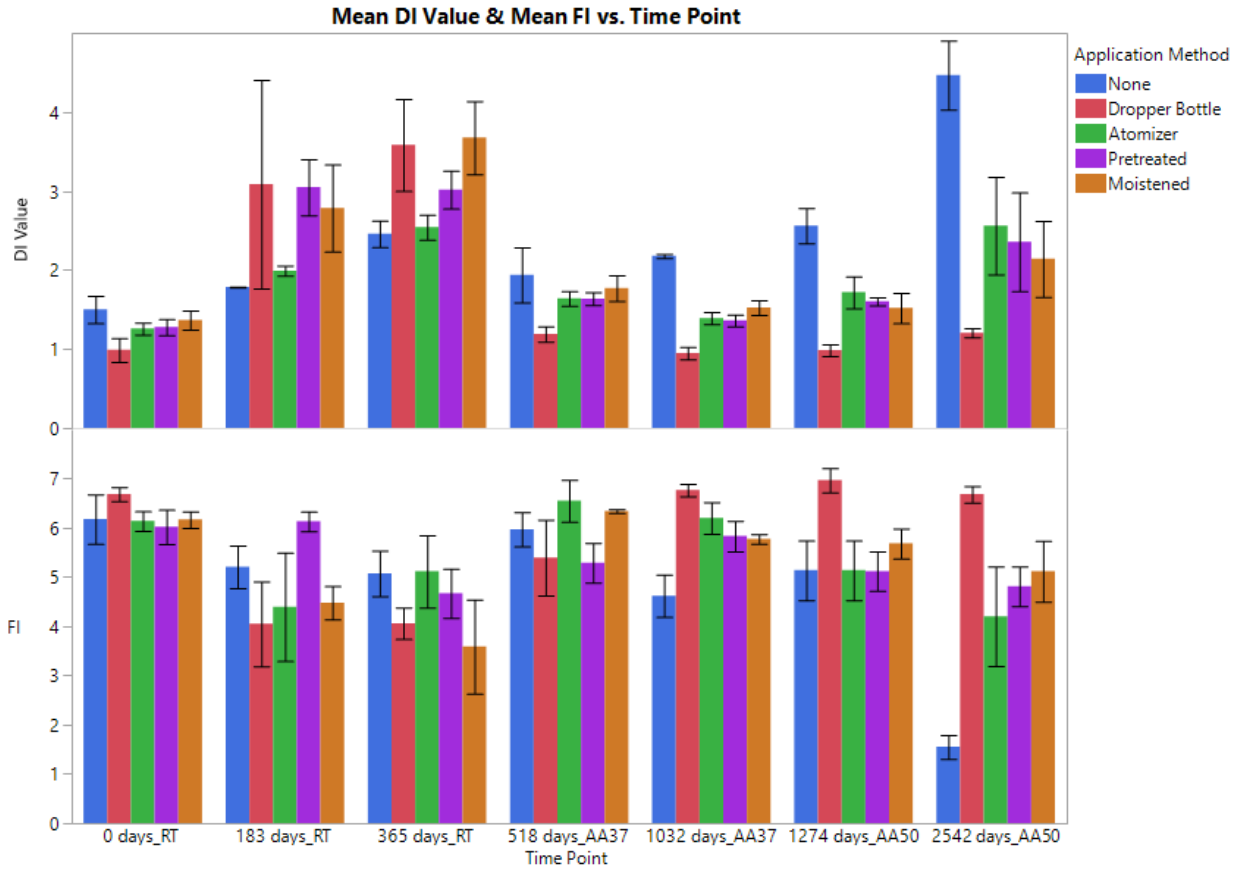

Figure 4: Mean DI and mean *FI* values obtained after applying EDTA to saliva swabs with the dropper bottle, atomizer, pretreated swab, and moistened swab preservative application methods. Nasal and oral spray means were calculated but are not shown. The untreated control samples are represented as "None." Each error bar is constructed using one standard deviation from the mean.

Table 7: Tukey HSD comparisons of mean DI & *FI* values resulting from dropper bottle, atomizer, pretreated swab, and moistened swab preservative application methods. Nasal and oral spray data were included in the statistical analysis but are not shown.

EDTA Application Methods Compared		Tukey HSD *p*-values													
		0 day		183 day		365 day		518 day		1032 day		1274 day		2542 day	
		DI	*FI*	DI	*FI*	DI	*FI*	DI	*FI*	DI	*FI*	DI	*FI*	DI	*FI*
None	Dropper Bottle	0.005*	0.356	0.136	0.279	0.021^	0.396	<0.001*	0.726	**<0.001***	**<0.001^**	**<0.001***	**0.010^**	**<0.001***	**<0.001^**
None	Atomizer	0.349	1.000	0.999	0.650	1.000	1.000	0.309	0.726	**<0.001***	**<0.001^**	0.006*	1.000	**0.004***	**0.024^**
None	Moistened	0.879	1.000	0.363	0.746	0.012^	0.087	0.845	0.951	**<0.001***	**0.007^**	<0.001*	0.849	**<0.001***	**0.002^**
None	Pretreated	0.44	0.991	0.155	0.511	0.487	0.976	0.298	0.568	**<0.001***	**0.005^**	0.002*	1.000	**0.002***	**0.005^**
Atomizer	Dropper Bottle	0.248	0.281	0.274	0.990	0.035^	0.355	0.044*	0.094	0.017*	0.357	**0.016***	**0.010^**	0.052	0.037^
Pretreated	Dropper Bottle	0.188	0.126	1.000	0.011*	0.481	0.854	0.046*	1.000	**0.027***	**0.034^**	0.051	0.009^	0.125	0.168
Pretreated	Atomizer	1.000	0.998	0.307	0.039*	0.654	0.962	1.000	0.058	1.000	0.777	0.995	1.000	0.998	0.970
Pretreated	Moistened	0.979	0.994	0.997	0.052	0.318	0.326	0.938	0.148	0.749	1.000	0.999	0.831	0.998	0.999
Moistened	Dropper Bottle	0.047*	0.336	0.993	0.970	1.000	0.949	0.007*	0.228	**0.002***	**0.024^**	0.116	0.097	0.291	0.327
Moistened	Atomizer	0.946	1.000	0.61	1.000	0.020*	0.075	0.944	0.997	0.878	0.663	0.918	0.849	0.933	0.830

* The application method listed first produced significantly higher values; $p < 0.05$
^ The application method listed second produced significantly higher values; $p < 0.05$

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References

1. Bonnet J, Colotte M, Coudy D, et al. Chain and conformation stability of solid-state DNA: implications for room temperature storage. Nucleic Acids Research. 2010;38(5):1531-1546.
2. Di Mambro VM, Azzolini AE, Valim YM, et al. Comparison of antioxidant activities of tocopherols alone and in pharmaceutical formulations. International Journal of Pharmaceutics. 2003;262(1-2):93-99.
3. Miki W. Biological functions and activities of animal carotenoids. Pure and Applied Chemistry. 1991;63(1):141-146.
4. Millar AD, Rampton DS, Blake DR. Effects of iron and iron chelation in vitro on mucosal oxidant activity in ulcerative colitis. Alimentary Pharmacology & Therapeutics. 2000;14(9):1163-1168.
5. Lindsay WL, Norvell WA. Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Science Society of American Journal. May 1978;42(3):421-428.
6. Dorn PL, Selgean S, Guillot M. Simplified Method for Preservation and Polymerase Chain Reaction-amplification of Trypanosoma cruzi DNA in Human Blood. Memórias do Instituto Oswaldo Cruz. 1997;92(2):253-255.
7. Warleta F, Quesada CS, Campos M, et al. Hydroxytyrosol protects against oxidative DNA damage in human breast cells. Nutrients. 2011;3(10):839-857.
8. Lykidis D, Van Noorden S, Armstrong A, et al. Novel zinc-based fixative for high quality DNA, RNA and protein analysis. Nucleic Acids Research. 2007;35(12):e85.
9. Bathrick AS, Mealy JL, Cavanaugh SE, et al. Long-term preservation of biological evidence with commercial off-the-shelf preservatives. Journal of Forensic Identification. 2017; 67(4):519-535.

10. Clermont D, Santoni S, Saker S, et al. Assessment of DNA encapsulation, a new room-temperature DNA storage method. Biopreservation and Biobanking. 2014;12(3):176–183.
11. Hemmerich KJ. General aging theory and simplified protocol for accelerated aging of medical devices. Medical Plastics and Biomaterials Magazine. 1998;5:16–23.
12. Hukins DWL, Mahomed A, Kukureka SN. Accelerated aging for testing polymeric biomaterials and medical devices. Medical Engineering & Physics. 2008;30(10):1270–1274.
13. Hedman J, Ansell R, Nordgaard A. Ranking index for quality assessment of forensic DNA profiles. BMC Research Notes. 2010;3:290.
14. Bathrick AS, Davoren JD. Accelerated Aging of Forensically Relevant Biological Materials on Swabs. Under review 2020.
15. Bathrick AS, Davoren JD. Preservation of Forensically Relevant Biological Materials with Commercial Off-the-Shelf Antioxidants and Chelators. Under review 2020.
16. Clark GS. Shelf Life of Medical Devices. Rockville, MD: Center for Devices and Radiological Health; 1991.
17. Bathrick AS, Davoren JD. Evaluation of Methods for the Application of Preservative Solutions to Saliva on Swabs. Poster made available online for Bode 2020 Virtual Forensic DNA Conference. October 26-27, 2020. https://bit.ly/2VBOA8K