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Optimization and Comparison of Methods for Separation of Spermatozoa from Superabsorbent Polymer-Containing Forensic Evidence

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Abstract

Sexual assault is currently one of the most prevalent crimes that affects victims of all ages. Forensic DNA analysts often confirm proof of contact in sexual assault cases through the identification of spermatozoa and subsequent STR profiling. Diapers and other feminine hygiene products, such as maxi pads and ultrathin pads, are types of superabsorbent polymer-containing evidence that complicate the process of DNA analysis due to the trapping of the spermatozoa in the gel-like matrix. In this study, a comparison of methods was performed to determine which extraction technique produces the highest sperm cell and DNA yield and generates a usable STR profile. When comparing the previously reported centrifugal filtration and calcium chloride ($CaCl_2$) dehydration methods, a teasing and filtration method, and a sodium chloride (NaCl) and filtration method, microscopic examination demonstrated significantly different sperm yields using the sodium chloride method for the diaper samples and the teasing and filtration method for the remaining substrate types. However, when quantifying the DNA extracts obtained from the substrates, most methods resulted in similar DNA concentrations, with little indication of degradation or inhibition. All four extraction methods produced full STR profiles with no indication of inhibition or degradation. This suggests that forensic laboratories have the flexibility to choose among these spermatozoa extraction methods when analyzing superabsorbent polymer-containing evidence.

Keywords: forensic science, superabsorbent polymers, semen, spermatozoa, diapers, sanitary napkins, DNA, sexual assault

Introduction

Biological evidence discovered in or on a sexual assault victim's body, or other items located at the scene, can provide proof of physical contact, and can connect an offender to the crime (1). The biological fluid most commonly present and searched for in these instances is semen, which is identified through the presence of spermatozoa (1, 2). Once sperm cells have been identified, the DNA can be extracted and analyzed using short tandem repeat (STR) amplification and subsequent fragment analysis to form a profile that can be compared to reference samples or to DNA databanks (3). Forensic laboratories have protocols and standards in place for the extraction of semen and spermatozoa for microscopic examination and subsequent DNA analysis from substrates such as swabs, clothing, and other commonplace materials. However, there is extraordinarily little guidance on separation of spermatozoa from evidence that contains superabsorbent polymers (SAPs), such as sanitary napkins and diapers (4).

The composition of feminine napkins and infant diapers are very similar, with the main difference being in how the product fastens into place for the wearer. These products consist of four layers designed for the prevention of leaks and the comfort of the wearer. The top layer is a thin, perforated, fluid permeable sheet of polypropylene and polyethylene that allows fluids to pass through to the inner layers of the pad or diaper (5). Next is an acquisition layer that may or may not be present in certain absorbent personal hygiene products. This layer contains cellulose and polyester and helps in the even distribution of fluids and the prevention of leakage. The core that lies below the top and acquisition layer is the main area where fluid is held (5). It is composed of cellulose fluff and superabsorbent polymer that can take in fluid up to thirty times its weight (5, 6). The final layer is fluid impermeable to prevent the leakage of the liquid being

held within the core. Feminine napkins typically contain adhesive on this bottom layer to attach to the clothing of the wearer (5).

The personal hygiene products previously mentioned that contain SAPs are effective because they can absorb and hold water and other liquids of great volumes (7). The SAPs are made up of cross-linked polymer networks of poly(acrylic acid), which with the absorption of liquid, becomes a gel with rubberlike consistency that prevents the leaking of the fluid even under mechanical pressure from the wearer (6, 7, 8). The swelling of the SAPs from a coiled formation to a 3-D structure is limited by the cross-linked composition and the forces of retraction, which allow the polymer to absorb to a finite amount without dissolving and not to an infinite dilution (7). These characteristics of SAPs are essentially why items of evidence containing them can be problematic. They not only trap the fluids they are intended for, but in cases where semen is present, they also hold it, and potential spermatozoa, within the core of the sanitary napkin or diaper. This core layer of the SAP-containing material is in fact where most of the biological evidence is located (9). However, most laboratories only use the top layer during extraction and DNA analysis, which can reduce the amount of DNA that is available to develop a STR profile (9, 10). The ability to extract the semen and spermatozoa from the core of the material could greatly improve the volume of sample available to be tested (11).

Understanding the value of the semen and spermatozoa that remain within the core of the SAP-containing products, experimental methods have been assessed in order to attempt to extract the biological fluid from the polymers. Early research by Giusti et al. analyzed semen deposited on sanitary napkins. The absorbent material was cut into smaller pieces, after the plastic was discarded, and then was placed in PBS with 2% Sarkosyl® at 4°C with agitation, was filtered through a nylon mesh, and then was centrifuged (12). This study, being one of the first to

extract semen from each layer of the sanitary napkin, exhibited that minor amounts of male DNA could be obtained from absorbent evidence (9 ,12). Hulme et al. tested water elution and Sperm Elution® methods to try to elute sperm from different fabric types, which included panty liners. The Sperm Elution® method is a two-phase technique where a combination of buffers is used to elute nucleated epithelial cells from the material to create an epithelial enriched fraction in phase one, and a sperm enriched fraction in the second phase. However, this resulted in no profile being developed with the water elution and a partial profile with the Sperm Elution® due to the absorbent quality of the hydrogel (13). Later, Camarena et al. were able to demonstrate that extraction of semen from the entire excision of the SAP-containing material resulted in an increase in DNA yield as compared to examining just the top-layer. The report also showed that the use of a centrifugal-filter device using a nylon fabric with TNE buffer added prior to centrifugation was a necessary step to separate the SAPs and pulp of the sanitary napkin or diaper. This resulted in better visualization of spermatozoa than if no filtration was utilized (14). Gregório et al. also provided evidence of the benefit of using the entire excision rather than just the top layer of the SAP-containing items. Utilizing chemical treatment (the best results being with the use of isopropanol), pressure shredding of the excision, and filtration through a nylon membrane, there was an increase in sperm and male cell recapture (11). O'Connor et al. introduced a SAPSWash method to extract and recover spermatozoa which involved incubation of the SAP-containing sample in 0.5 M calcium chloride ($CaCl_2$) followed by centrifugation in a spin basket. This method led to the "dewaterisation" of the hydrogel, which resulted in recovery of enough spermatozoa, albeit poor, to develop a full STR profile. However, this experiment involved a lengthy sample preparation time of overnight freezing or air drying and an additional hour-long incubation time in the $CaCl_2$, resulting in relatively low spermatozoa yields (15).

Chen et al. published a classroom activity that demonstrates the ability of sodium chloride (NaCl) to cause the release of water from superabsorbent material (16). As mentioned previously, when the superabsorbent polymer is exposed to water, it becomes a swollen gel-like consistency (6, 7, 8, 16). Once the interior state of the gel and the exterior solution has reached an equalized osmotic pressure, an equilibrium can be reached. When a monovalent metal ion, like NaCl, is added to the gel substance, the ionic strength outside the gel increases and the water inside the gel will diffuse out. Furthermore, the NaCl ions diffuse into the gel which helps to collapse the gel further and prevent the gel from being able to reabsorb the water (16). This technique of adding crystallized NaCl to the superabsorbent polymer-containing material has not been evaluated on forensic evidence but could have the potential to draw out sperm cells along with the water when diffusion is occurring. This method could have the potential to increase the spermatozoa yield, which will also increase the amount of DNA available for analysis and profile development.

While there is a push to reduce the number of untested sexual assault kits (SAKs) in the United States, greater than 200,000 kits remain untested (17). The evidence that is submitted within these kits come in many forms; however, many laboratories choose to only test a few of the samples submitted. Usually, the most probative items are chosen (17). While evidence composed of SAPs are not frequently encountered in forensic laboratories, which could be due to submitting agencies realizing that these products have historically been challenging sample types with low yields, they are commonly used in everyday life. Diapers are generally worn throughout the first few years of an infant's life, and typical menstrual periods for women are around seven days long and occur every 28 days. Without a standard protocol for the evaluation of SAP-containing evidence, these items that are so commonly used may not be selected due to the

potential of the low recovery of spermatozoa or even inhibition from the SAPs. The optimization of an efficient technique to increase DNA recovered from feminine napkins or diapers could help provide even more options for the testing of sexual assault samples to help decrease the backlog of SAKs (17).

Materials and Methods

Sample Preparation

Practicing the ethical standards put in place by the institutional research board (IRB), semen samples were voluntarily collected by human donors, and were stored at -20°C prior to use. The semen was prepared before each analysis as a 1:5 dilution using 1x PBS (Phosphate-buffered saline) as the diluent, to avoid any freezing and thawing effects that can cause the sperm cells to clump together.

Three SAP-containing substrates were analyzed: Always® Ultra Thin pads and Maxi pads (Procter & Gamble, Cincinnati, OH) and Up & Up™ infant diapers (Target Corporation, Minneapolis, MN). The substrate samples were prepared by excising a disk from the substrates with the diameter of approximately 6.35 mm using a sterile hole puncher. The diluted semen was then added to the top layer of each substrate and allowed to dry for 10 minutes prior to spermatozoa extraction for sperm cell yield comparison, and 24-48 hours prior to spermatozoa extraction for DNA analysis and DNA yield comparison. The quantity of DNA added to the substrates varied for each comparison. For the diapers and maxi pads, 100 µL of 1:5 diluted semen was added to the diaper throughout the experiment. However, for the ultra thin pads, the full volume was not absorbed when doing the microscopic examinations using the centrifugal-filtration and SAPSWash extraction methods. Due to the lower absorbency of the ultra thin

substrate, the original volume of 100 µL was reduced to 50 µL for the remaining two methods in the sperm cell yield comparison and for all four methods prior to DNA analysis.

Liquid 1:5 semen was used as a positive control for comparison in the microscopic examination of spermatozoa. Two positive controls were also created for DNA analysis and yield comparison which consisted of four replicates of 100 µL of 1:5 semen pipetted directly onto a cotton swab, and three replicates of 100 µL of liquid 1:5 semen. The samples on the cotton swabs were allowed to dry for 24-48 hours prior to DNA extraction.

Spermatozoa Extraction: Centrifugal-Filtration with TNE Extraction Method

The centrifugal filtration method by Camarena et al. was performed by sandwiching a 3 x 3 cm square of 20 x 25 µm mesh nylon fabric, purchased from a fabric store, between a spin basket (Promega, Madison, WI) and a 2 mL microcentrifuge tube. 400 µL of TNE (0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA) buffer was added, immediately followed by the centrifugation of the samples for ten minutes at 2000 x g using a Sorvall™ Legend™ Micro 17 Microcentrifuge (Thermo Scientific, Waltham, MA) (14). The spin baskets and filters were removed, and the substrates discarded, leaving the spermatozoa extract.

A few techniques were evaluated to optimize and increase the spermatozoa yield of this method. The methods analyzed included adding a 1-hour incubation of the substrate in TNE prior to filtration at different temperatures (22°C, 37°C, 56°C, 95°C) to attempt to disrupt and degrade the SAP gel. However, no consistent increase in yield was seen, so the original method was used for comparison during this experiment.

Spermatozoa Extraction: SAP Sperm Wash (SAPSWash) Extraction Method

The SAPSWash method by O'Connor et al. was tested by placing the substrates, previously prepared, into 1.5 mL microcentrifuge tubes and incubating them for one hour in 1 mL of 0.5 M $CaCl_2$ at room temperature (15). The solid substrates were transferred to 1.5 mL tubes containing a spin basket and were essentially discarded without trying to extract any extra liquid sample. The liquid samples from the incubation step were then transferred to additional 1.5 mL microcentrifuge tubes and were centrifuged for one minute at 15,000 rcf. After centrifugation, the supernatant was removed and discarded, and the pellet was resuspended two more times in 1 mL of 0.5 M $CaCl_2$, with centrifugation following each resuspension. The supernatant was again removed and discarded, and the pellet was resuspended in 1 mL of ddH_2O followed by centrifugation. The supernatant was removed a final time and the pellet resuspended in 100 µL of ddH_2O (15).

Again, a few techniques were evaluated in an attempt to optimize and increase the spermatozoa yield of this method. The methods analyzed included a variety of different techniques such as testing different temperatures (22°C, 37°C, 56°C, 95 C) during the 1-hour incubation in $CaCl_2$ to disrupt and degrade the SAP gel, changing the 1.5 mL tubes to 2 mL tubes, adding a centrifugal filtration step after the incubation step, adding shaking to the incubation step, exchanging the ddH_2O wash step for a TNE wash step, and other small variations. However, no consistent increase in yield was observed for any of the changes made, so the original method was used for comparison during this experiment.

Spermatozoa Extraction: Sodium Chloride (NaCl) and Filtration Method

During the sodium chloride (NaCl) and filtration method, the prepared substrates were placed into 2 mL microcentrifuge tubes and 500 µL of ddH_2O was added. 0.315 g of NaCl, was added to the tubes containing the substrates and the contents of the tubes were stirred to allow

the salt and substrate to interact. The liquid was then transferred to additional 2 mL tubes containing 3 x 3 cm squares of 20 x 25 µm nylon fabric placed beneath spin baskets (Promega). To try to liquify any remaining gel in the first tube, an additional 0.05 g of NaCl was added and the contents of the tubes were stirred and transferred to the 2 mL tubes containing the nylon fabric and spin basket. The samples were centrifuged through the filter for five minutes at 10,000 rpm (9600 x g), which are typical centrifugation parameters used in forensic laboratories to pellet sperm cells. The filters, spin baskets, and substrates were discarded, and the supernatant was removed, leaving the sperm pellet. The sperm pellet was washed two more times using 400 µL of ddH_2O, with centrifugation after each resuspension. Following the wash steps, the supernatant was removed, and the sperm pellet was resuspended in 100 µL of ddH_2O.

Several techniques were assessed in the optimization of this method which included different quantities of ddH_2O, different quantities of NaCl, a 5 M solution of NaCl instead of crystallized NaCl, a supersaturated 50% w/v solution of NaCl, 1x PBS in place of ddH_2O, and other variations of this method. The method described above produced the most consistent and highest sperm cell yields, and therefore was used for comparisons.

Spermatozoa Extraction: Substrate Teasing and Filtration Method

Due to the low SAP content of the maxi pad and ultra thin samples, a new method was developed utilizing only water and manual separation of the substrate in an attempt to release the sperm cells. The prepared substrates were placed into 2 mL microcentrifuge tubes and 400 µL of ddH_2O was added. The substrates were immediately teased apart with tweezers until broken up (about ten seconds) and then transferred to 2 mL tubes containing 3 x 3 cm squares of 20 x 25 µm nylon fabric placed beneath spin baskets (Promega). The tubes were centrifuged for five minutes at 10,000 rpm and the substrates, filters, and spin baskets were discarded.

Evaluation of Spermatozoa Yield

For comparison of methods utilizing sperm cell yield, 6 diaper replicates, 6 ultra thin replicates, and 5 maxi pad replicates (n = 17) were tested using the centrifugal-filtration method; 3 replicates per substrate (n = 9) were tested using the SAPSWash method and NaCl/filtration method; and 4 replicates per substrate (n = 12) were used for the teasing/filtration method. A new positive control of liquid 1:5 semen was prepared, and used immediately, each time an extraction technique was tested to minimize the negative effects of freezing and thawing (clumping of sperm cells) the diluted control. During the optimization of the methods, and the comparison of the sperm cell yields, semen samples from 4 donors were used.

Following the spermatozoa extraction methods, release of sperm cells was assessed by preparing a microscope slide in which 4 µL of the sample was stained with Kernechtrot-Picroindigocarmine (KPICS). Four random fields of view were analyzed under 400X magnification, and the total number of sperm heads was counted per field and averaged together to determine the percent yield of spermatozoa. During the optimization of the methods, a positive control of liquid 1:5 diluted semen was prepared and analyzed for comparison and for determination of recovery percentage (14). The following formula was used for percent yield calculations:

$$Percent\ Yield = \frac{average\ count\ of\ sperm\ heads\ for\ test\ sample}{average\ count\ of\ sperm\ heads\ for\ liquid\ \ 1{:}5\ semen} \times 100\%$$

For the methods that resulted in a 400 µL extract, the average count of sperm heads for the test sample was multiplied by 4 to account for the larger sample volume and subsequent 4-fold dilution. Furthermore, whenever only 50 µL of 1:5 semen was added to the ultra thin samples, the average count of sperm heads for the test sample was multiplied by 2 to normalize

the percent yield comparisons between the substrates that received 100 µL of 1:5 semen. If the sample resulted in a 400 µL extract and had only 50 µL of 1:5 semen applied, then the sperm head count was multiplied by 8.

DNA Analysis

For comparison of SAP extraction methods using DNA quantification data, 5 replicates per substrate per method (n = 60) were analyzed. 4 samples of untreated and unfiltered 1:5 semen on cotton swabs and 3 samples of 100 µL liquid 1:5 semen were also tested as controls. All the samples and positive controls for DNA analysis were created from the semen samples provided by 1 donor. Prior to DNA extraction, all spermatozoa extraction samples and liquid 1:5 semen positive controls were centrifuged for five minutes at 5,000 rpm to pellet the sperm cells. All but about 50 µL of the supernatant was pipetted out of the tube and the sperm pellet was resuspended with lysis buffer as described below.

A manual DNA extraction was performed using the QIAGEN QIAamp® DNA Investigator Kit (Qiagen, Hilden, Germany). Manufacturer recommendations were followed, with the addition of 20 µL 1 M dithiothreitol (DTT) to extract DNA from the spermatozoa, and all samples had a final elution volume of 50 µL. The samples were quantified using the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, Waltham, MA) with the ABI Prism 7500 Sequence Detection System using half volume reactions. The data from this quantification were analyzed using HID Real-Time PCR Analysis Software (Applied Biosystems), and evaluated not only for quantity, but also evidence of inhibition and degradation using the IPC and degradation index features of this method. The 1:5 semen on cotton swab samples were used as positive controls in the DNA yield comparison to compare the yields

received from the SAPs samples to yields that would most likely be seen in forensic laboratories. Percent yield of DNA was calculated using the following formula:

$$Percent\ Yield = \frac{DNA\ concentration\ of\ sample\ (ng/\mu L)}{DNA\ concentration\ of\ 1{:}5\ semen\ on\ cotton\ swab\ (ng/\mu L)} \times 100\%$$

DNA concentrations were averaged across replicates for each method, and the replicate closest to the average was selected for STR amplification. 1 replicate per substrate per method (n = 12), along with 4 samples of 1:5 semen on cotton swabs and 4 reagent blanks were analyzed. GlobalFiler™ PCR Amplification Kit (Applied Biosystems) was used to amplify 0.5 ng of DNA per sample on the ProFlex PCR System (Applied Biosystems), utilizing an initial incubation at 95°C for 60 seconds, a 29-cycle amplification, and a final extension at 60°C for ten seconds. Amplicons were separated and detected using an ABI 3500 Genetic Analyzer (Applied Biosystems) and a 1.2 kV, 15 second injection. Data analysis was completed using GeneMapper™ ID-X Software v1.6 (Applied Biosystems) with a 100 relative fluorescent units (RFU) threshold. The allele peak heights for each sample were then averaged and compared utilizing 37 alleles from 1 STR profile.

Data Analysis

A one-way analysis of variance (ANOVA) was performed on the sperm cell yield and DNA yield to determine if there was a statistical significance ($\alpha = 0.05$) between the methods used for spermatozoa extraction for each substrate type. A Tukey's multiple pairwise honest significance difference (HSD) test was then used to determine the significant differences of means between the methods. All statistical tests were performed using the Past 4.02 scientific data analysis software platform (18). No statistical tests were performed for the comparison of average peak heights due to the analysis of only 1 replicate.

As mentioned previously, potentials for inhibition and degradation were evaluated prior to amplification using the Quantifiler™ Trio Quantification Kit. The internal PCR control (IPC) cycle threshold (C_T) values of the samples were analyzed to determine if they fell within a 26-29 C_T range, suggested by a validated protocol, indicating no inhibition present within the sample. According to the protocol, less than 26 or more than 29 C_T values could indicate that inhibition was present (19). The degradation index, which represents the ratio of the small autosomal DNA concentration to the large autosomal DNA concentration, was also evaluated for each sample to indicate if any degradation had occurred during the analysis. A degradation value below one indicates that no degradation occurred, and values of 1-4 indicate minimal degradation. (19). Finally, the averages of the IPC values and degradation index ratios were calculated for the replicates of each substrate type and method.

Results and Discussion

Evaluation of Spermatozoa Yield

The previously published centrifugal-filtration and SAPSWash methods were first evaluated to determine if there were steps within the methods that could be optimized to increase spermatozoa yields. Various techniques were analyzed, including varying temperatures during incubation steps, adding shaking, adding filtration, etc. However, no technique produced a consistent increase in sperm cell yield. Therefore, the originally published methods were used for comparison. Both methods produced a consistent low sperm cell yield across all three substrates, with the SAPSWash method producing average yields less than 5% and the centrifugal-filtration method less than 28% (Fig. 1). These results are comparable to the yields obtained by Camarena et al., but the SAPSWash method by O'Connor et al. produced consistently miniscule sperm counts, and often no yield was detectable (14, 15). Additionally, the centrifugal-filtration method

created a cloudy microscopic field-of-view that made it difficult to visualize and count sperm heads. Both methods also demonstrated trouble breaking down the gel and resulted in gel particles dispersed on the microscope slide that trapped clumps of sperm cells. Because of these results, additional methods were developed and tested in an attempt to increase the sperm cell yield and minimize the effects of the superabsorbent polymer gel.

The NaCl/filtration method was developed in an attempt to promote the gel to release the water and sperm cells through diffusion. This method produced significantly different means compared to the other methods for the diaper samples with an average over 65% spermatozoa yield. This is believed to be due to the higher concentration of SAP contained within the diaper. When the fluid was added to the dry substrate, the gel absorbed the entire volume. With the addition of NaCl, the fluid, and thus also sperm cells, were released from the gel at a greater quantity than previously seen and the gel greatly reduced in size. This method also created a clearer microscopic field-of-view with less fragments of gel present on the slide.

Even with the increased sperm cell yield in the diaper, the ultra thin pad samples and maxi pad samples still produced low yields with the NaCl method. When visualizing the substrates with the naked eye and under a microscope, it was evident that there was less SAP present within these substrate types as compared to the diapers. The ultra thin sanitary napkins are designed to be low profile, which results in lower absorption. Thus, they have lower quantities of SAPS, while the maxi pads appeared to be composed of wood pulp. Due to the lower amount, or absence, of SAP in these substrates, utilizing a method similar to methods used for the microscopic visualization of spermatozoa when the substrate is a cotton swab (the addition of water and teasing) could increase the sperm cell yield. Confirming these postulations, the teasing/filtration method resulted in an increase in the average spermatozoa yield for the

maxi pad (48%) and the ultra thin pad (over 100%), as compared to the other three methods, and created a clear field-of-view for visualization. When comparing the yields for the maxi pad, this method resulted in significantly different means compared to the NaCl/filtration and SAPSWash methods but did not show a significant difference from the centrifugal-filtration method. However, for the ultra thin pad, this method produced significantly different means than every other method tested.

It should be mentioned that the spermatozoa yield could vary from one random field-of-view to the next. Depending on what field-of-view is chosen, different counts could be made from individual to individual. This subjectivity is due to the randomization of the area of the slide chosen, and the distribution of the sperm cells. Some methods can concentrate the sperm cells in gel or even around the outer edge of the drop made on the slide. Due to this variability, further DNA analysis was performed to provide a more accurate comparison of the methods.

DNA Yield Comparison

To measure how many sperm cells, and thus DNA, were extracted from the substrate, DNA extracts from the spermatozoa extractions were quantified (Table 1) and the percent DNA yields were calculated. The centrifugal-filtration method produced the highest yield for the diaper and ultra thin pad samples, with a statistically significant difference when extracting sperm cells from the ultra thin pads (Fig. 2). The centrifugal-filtration method and the teasing/filtration method were not significantly different for the diapers. For the maxi pad samples, the teasing/filtration method again demonstrated a higher yield (55%) and significantly different means than the NaCl/filtration and SAPSWash methods. However, the means were not significantly different from the centrifugal-filtration method. Ultimately, the SAPSWash method

had the lowest DNA yield, demonstrating that it performed the poorest across all substrates. O'Connor et al. similarly reported low yields of spermatozoa and thus low yields of DNA (15).

Inhibition and Degradation Evaluation

To evaluate whether any of the spermatozoa extraction methods would cause inhibition to further downstream analyses, and to determine if the DNA had been degraded during earlier steps, the IPC C_T value and the degradation index of each sample were examined. Potential issues could have arisen due to SAPs being left in the extraction sample, an excess of salt remaining in the sample, or too vigorous of stirring or teasing could have occurred. Camarena et al. previously reported that no inhibition was seen when evaluating the centrifugal-filtration method, and O'Connor et al. stated that the SAPSWash method demonstrated signs of inhibition when visualizing the STR profiles (14, 15). However, no pattern or major signs of inhibition or degradation was observed amongst any of the samples regardless of method or substrate type. The largest deviation from the IPC cycle threshold range that indicates no inhibition (26-29) was only half a cycle longer, indicating very low inhibition. The average IPC cycle threshold value for almost all substrates and methods fell within the cycle range indicating no inhibition (Table 1). Any degradation index ratio above 1 would indicate degradation is present. However, the highest ratio seen was only 1.86, indicating very low levels of degradation. The average degradation index ratio was calculated for each substrate and each method, and most values were only slightly above 1 with the highest average degradation index radio being 1.32. The data demonstrates that none of the methods tested show signs of degradation or inhibition that would affect downstream DNA analysis.

STR Profile Comparison

The ultimate goal for forensic evidence samples is to produce an STR profile that can be compared to reference samples or can be uploaded into DNA databanks. To test whether or not the spermatozoa extraction methods could produce profiles, one replicate per substrate per method was taken through the entire DNA analysis process. In this final analysis, all 12 samples produced a full STR profile without any signs of inhibition or degradation. The high molecular weight loci showed no signs of allele dropout or low peak height, which could be signs of the ski slope effect of preferential amplification based on locus size. These results are similar to the findings of Camarena et al. who also demonstrated full profile development for their samples (14). However, O'Connor et al. had to implement a clean-up step for some samples to eliminate inhibition and allele dropout (15).

When comparing the average peak heights for the diaper samples, the NaCl/filtration method had a peak height average over twice the other methods (Fig. 3). Except for the SAPSWash method on the maxi pad, the ultra thin and maxi pad samples showed similar peak height averages across all extraction methods. These results indicate that the NaCl/filtration method is beneficial when analyzing evidence with higher SAP content; however, any of the four methods can be utilized when analyzing substrates with lower SAP content. The benefit of all four methods being able to produce full STR profiles, is that the forensic scientist has the flexibility to choose which method to extract spermatozoa cells from superabsorbent polymer-containing evidence.

Overview of Results

To summarize the findings of the methods evaluated per substrate type, the results from the analyzed measurements were compared to show which methods produced acceptable or

usable outcomes (Table 2). For spermatozoa yield and microscopic examination, only one method per substrate produced satisfactory results. The NaCl/filtration method had the highest sperm cell yield and clearest field of view for the diaper samples, while the teasing/filtration method worked the best for the ultra thin and maxi pads. When analyzing the DNA yields and DNA concentrations obtained from each substrate, the SAPSWash method was the only method that produced poor results. Finally, all four methods generated full STR profiles with average peak heights that were easy to interpret and showed no signs of inhibition or degradation. These results indicate that a protocol could be developed for the analysis of SAP-containing evidence utilizing one or more of these methods based on the needs of the laboratory.

Conclusion

Superabsorbent polymer-containing evidence can produce valuable findings in sexual assault cases. Consequently, forensic laboratories should seek to establish a protocol that utilizes the entire content of the evidence to extract the highest amount of DNA possible. This study demonstrated that when dealing with the entire thickness of the sample, substrates with a higher content of superabsorbent polymer present may benefit from the use of the NaCl/filtration method, while substrates with a lower content of SAP can employ the simpler, less laborious methods of centrifugal-filtration or teasing/filtration. With that being said, all four methods analyzed produced full STR profiles with no evidence of inhibition or degradation. These findings give forensic DNA analysts the flexibility of choosing which method will suit their laboratories' workflow the best. The centrifugal-filtration and the teasing and filtration methods are rapid, require minimal reagents, and are not as hands-on as the other two methods. However, the NaCl/filtration method may be beneficial for certain types of SAP-containing materials. With

a protocol in place that takes advantage of the whole sample, DNA yield can be maximized, and more cases can be analyzed further reducing the sexual assault kit backlog.

Future work to provide more evidence of the effectiveness of these techniques is in progress. Currently, forensic laboratories only use the top layer of the substrate when examining SAP-containing evidence. For this reason, a top-layer DNA analysis of each of the substrates is being evaluated to compare to the DNA analysis of the entire excision. Moreover, various manufacturers of SAP-containing diapers and feminine hygiene products are being contacted to potentially gain insight into the composition of the substrates. Understanding the SAP content of the different products could assist in determining which spermatozoa extraction method should be applied to produce the greatest sperm cell and DNA yields.

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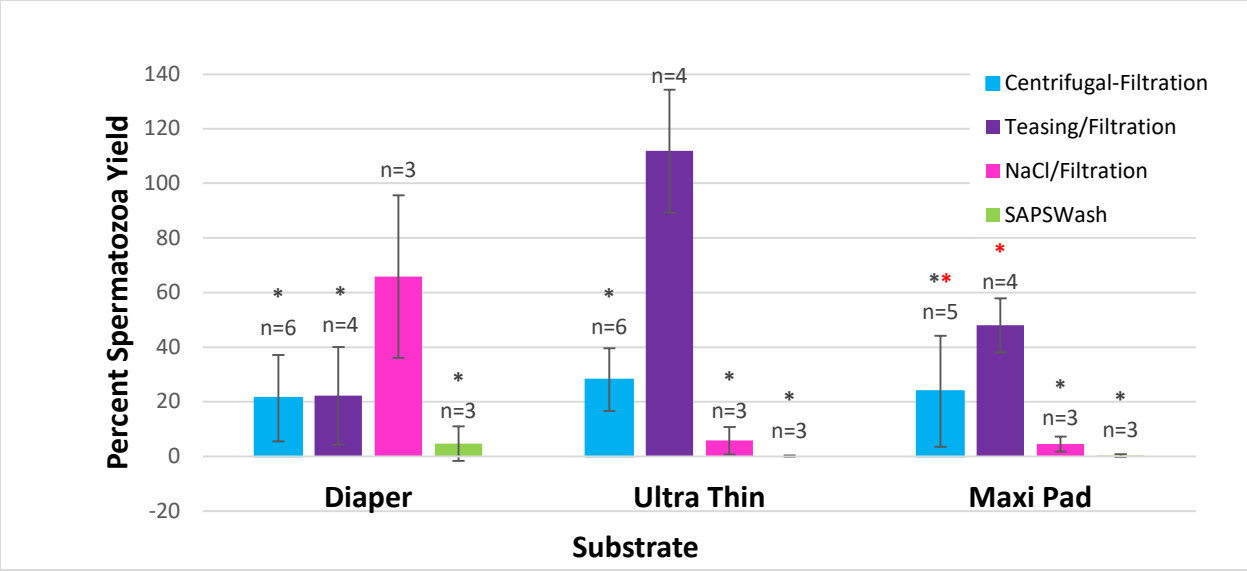

Figure 1: Comparison of Spermatozoa Yield Between Substrates and Extraction Method. Average sperm cell yield of each sample was calculated by dividing the sperm head count of the sample by the sperm head count of the 1:5 semen positive control and multiplying by 100%. The error bars represent the standard deviations of the measurements. * and * indicate methods that have spermatozoa yield means that are similar to each other or are not statistically different from each other.

Table 1: Comparison of average DNA concentration, IPC C_t values, and degradation indexes for each substrate and each method.

Substrate	Method	DNA Concentration (ng/µL)	Standard Deviation	IPC C_t Value	Standard Deviation	Degradation Index	Standard Deviation
Diaper (100 µL)	**TNE**	3.49	0.58	28.84	0.44	1.19	0.23
	Teasing	3.02	1.16	28.78	0.27	1.33	0.27
	NaCl	1.72	0.42	28.99	0.19	0.86	0.17
	SAPSWash	0.27	0.08	28.07	0.40	1.03	0.10
Ultra Thin Pad (50 µL)	**TNE**	2.14	0.44	28.81	0.36	1.00	0.17
	Teasing	1.52	0.28	28.62	0.30	1.04	0.11
	NaCl	0.90	0.17	28.26	0.20	1.30	0.22
	SAPSWash	0.32	0.28	28.08	0.21	1.12	0.18
Maxi Pad (100 µL)	**TNE**	2.40	0.87	28.94	0.43	1.03	0.49
	Teasing	4.01	1.73	29.06	0.12	1.05	0.13
	NaCl	1.42	0.66	28.81	0.25	1.08	0.41
	SAPSWash	0.75	0.31	28.12	0.17	1.05	0.14

Samples were quantified using the Quantifiler™ Trio DNA Quantification Kit with the ABI Prism 7500 Sequence Detection System using half volume reactions. The data from this quantification were analyzed using HID Real-Time PCR Analysis Software and were evaluated for quantity and evidence of inhibition and degradation using the IPC C_t values and degradation index features of this method.

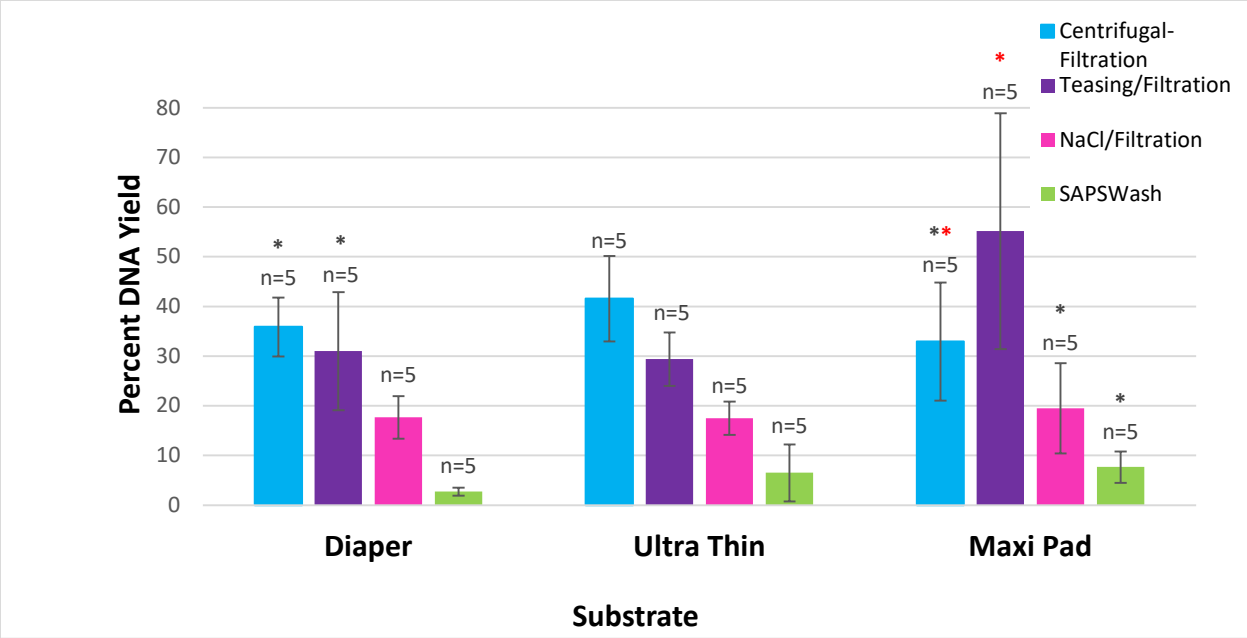

Figure 2: Comparison of Percent DNA Yield Between Substrates and Extraction Methods. The DNA yield of each sample was calculated by dividing the DNA concentration (ng/µL) of the sample by the DNA concentration (ng/µL) of the 1:5 semen on cotton swab positive control and multiplying by 100%. The error bars represent the standard deviation of the measurements. * and * indicate methods that have spermatozoa yield means that are similar to each other or are not statistically different from each other.

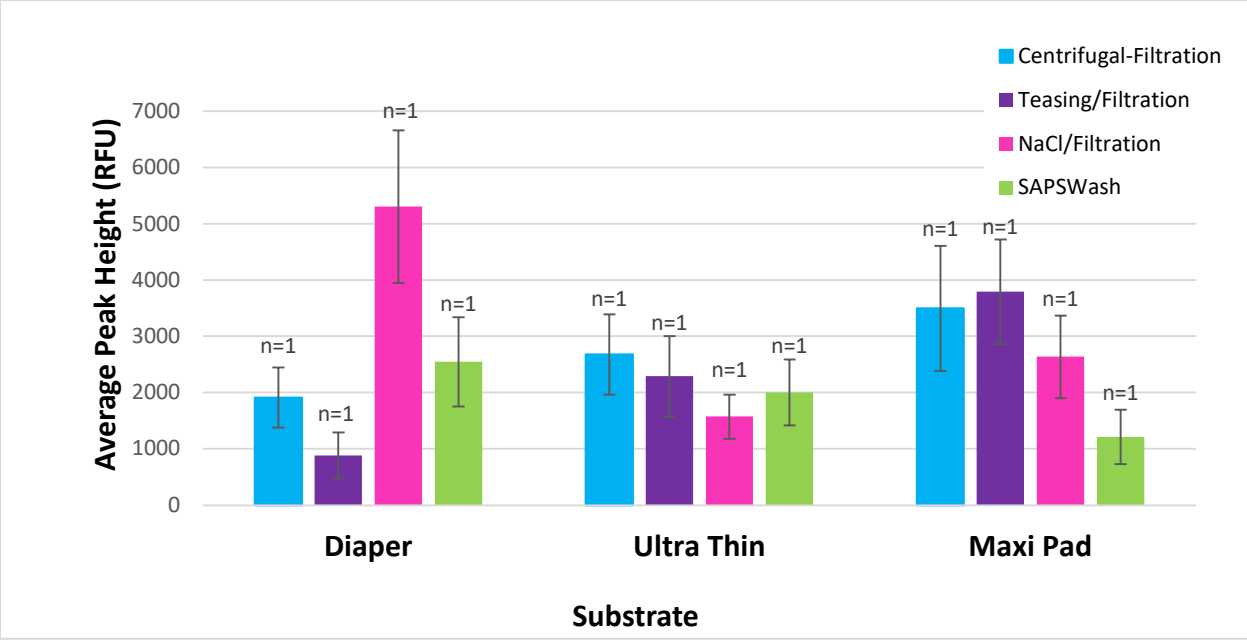

Figure 3: Comparison of Average Peak Heights Between Substrates and Extraction Methods. (n = 37 alleles from 1 STR profile.) GlobalFiler™ PCR Amplification Kit was used to amplify 0.5 ng of DNA per sample on the ProFlex PCR System. Amplicons were separated and detected using an ABI 3500 Genetic Analyzer. The error bars represent the standard deviations of the measurements.

Table 2: Acceptability of the methods for each criteria analyzed.

Substrate	Method	Percent Sperm Yield	Percent DNA Yield	Average Peak Height	Inhibition	Degradation
Diaper	TNE	×	✓	✓	✓	✓
	Teasing	×	✓	✓	✓	✓
	NaCl	✓	✓	✓	✓	✓
	SAPSWash	×	×	✓	✓	✓
Ultra Thin Pad	TNE	×	✓	✓	✓	✓
	Teasing	✓	✓	✓	✓	✓
	NaCl	×	✓	✓	✓	✓
	SAPSWash	×	×	✓	✓	✓
Maxi Pad	TNE	×	✓	✓	✓	✓
	Teasing	✓	✓	✓	✓	✓
	NaCl	×	✓	✓	✓	✓
	SAPSWash	×	×	✓	✓	✓

✓ = acceptable

× = not acceptable

Each method per substrate was evaluated for its ability to produce results that are acceptable for each category analyzed: percent sperm yield, percent DNA yield, average peak height, inhibition, and degradation.

Vita

Hannah Grace Wells was born on August 31, 1994, in Richlands, Virginia. She graduated from Richlands High School, Richlands, Virginia in 2012. She received her Bachelor of Science in Biological Sciences from Virginia Polytechnic Institute and State University, Blacksburg, Virginia in 2015, graduating Magna Cum Laude. She received a Master of Science in Forensic Science with a concentration in forensic biology from Virginia Commonwealth University in 2022.