The development of a rapid and reliable method (SAPSWash); for the extraction and recovery of spermatozoa from superabsorbent polymer containing products.

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Highlights

- Release of spermatozoa from absorbent sanitary towels and nappies / diapers
- Simple, rapid method to break down superabsorbent polymers to release spermatozoa
- A range of different sample types and storage conditions were evaluated
- DNA profiles obtained from spermatozoa released from superabsorbent polymers

Abstract

This work presents a rapid and reliable method to recover spermatozoa from Super Absorbent Polymers (SAPs) commonly found in sanitary protection products such as nappies and sanitary towels. The use of salt solutions was investigated and a protocol was developed using a calcium chloride ($CaCl_2$) solution to release semen deposited onto a selected SAP containing product. The method was tested on ultra-sanitary towel samples treated with a known amount of semen. A range of treatments were examined some samples were prepared and immediately frozen for storage and others were allowed to air dry overnight to replicate the condition of similar items recovered for examination in sexual offence cases. The method allowed the collection of low yields of spermatozoa, but these were still sufficient for microscopic identification of intact heads and to obtain ESI17 DNA profiles from all the samples. This report presents the method, the results obtained and discusses prospective adaptations to the method for validation to implement the method into forensic casework.

1. Introduction

Location, identification and recovery of spermatozoa form the major part of the forensic investigation of rape and many other sexual assault cases. This has been achieved historically by simple water extraction of typical exhibits, such as intimate swabs and clothing, thus releasing a mixture of semen and epithelial cells which can sometimes be further separated to facilitate DNA analysis. Several methods were reviewed and compared by Allard et al., (2007) [1]. Since then, many forensic service providers have developed their cell harvesting protocols by introducing more efficient recovery techniques, with examples such as the Sperm Elution™ method [2]. These methods include soaking substrates such as swab heads in buffer solutions with the addition of agitation and incubation steps to encourage the release of the spermatozoa from within the fibres of the swabs to generate an increased yield. Owers et al., (2018) [3] analysed casework data produced using the Sperm Elution™ method to provide Time Since Intercourse (TSI) information relevant to the improved sperm recovery. However, the superabsorbent polymers (SAPs) used in the manufacture of sanitary protection products, such as nappies and sanitary towels have been specifically designed to retain organic material deposited in them. Commercial research and development drives improvements in these polymers and products are regularly advertised as "new and improved" as the technology advances. SAPs form a gel network when they come into contact with liquids. The gel is not itself water soluble, but has the capacity to absorb high volumes of fluid [4]. Although water extraction is feasible on the upper surfaces of SAP containing items, the recovery from these layers can be limited and there was no existing method to release spermatozoa from within the SAP gel where the majority of the liquid sample was likely to have been absorbed. A method to release urine from nappies using $CaCl_2$ powder was described by Liu *et al.*, (2012) [5] and further studies by Hu *et al*., (2004) [6] had used a similar technique after considering varying concentrations of four different salts, achieving maximum urine recovery using calcium chloride dihydrate. They described that when in its dry state, the polymer chains of the SAP are coiled but they uncoil with the introduction of water. The water invokes a hydration process, causing the detachment of the sodium ions and creating negatively charged carboxyl groups which repel each other, forcing the chain to uncoil. It is this process which makes the polymer form a three-dimensional gel, supported by weak cross linking as it continues to absorb water. They

further observed that the SAP gel can be made to collapse in the presence of some metal cations (such as Ca^{2+}) as these bi-valent ions neutralise the poly-anions and bind the carboxylate groups causing the hydrophobic polymer backbone to collapse. It is these unique properties that led to the use of SAPs in a wide range of applications, not least sanitary protection products [4]. These properties however, also mean that spermatozoa within semen are trapped within the gel when deposited onto SAP containing forensic casework exhibits.

Camarena *et al.,* (2017) [7] published An Optimized Centrifugal Method for Separation of Semen from Superabsorbent Polymers for Forensic Analysis which sought to filter semen stained nappy pulp through non-commercial fabric filters using a buffered salt solution. This method, published whilst this SAPSWash study was underway, also considered the need to isolate any semen from the sanitary protection product pulp and encountered similar issues with residual SAP in their sample products and low spermatozoa yield. Gregorio *et al.,* (2019) [8], published after the SAPSWash study was complete, forged a similar approach by attempting chemical and physical shredding of semen containing SAP samples and measuring percentage recovery of spermatozoa, concluding that methods which promote the "dewaterisation" of the gel should be pursued.

Method development for this SAPSWash study comprised a number of experiments to optimise a protocol for the recovery of spermatozoa, using a source of boar semen. Early considerations included how to separate any released spermatozoa from the sanitary protection product wadding pulp, the significance of the number of salt washes and the most effective salt concentration to deliver maximum gel breakdown and minimising the number of sample handling steps to ensure maximum yield. The method development (which formed part of a wider study) was evaluated by counting the number of recovered spermatozoa and comparing these results to optimise the method. The development work and its results are not presented in detail in this paper and are discussed only to introduce the optimised SAP Sperm Wash (SAPSWash) method which was developed to optimise the breakdown of the SAP to release the spermatozoa before attempting to separate them from the pulp material. This work additionally sought to consider whether the method was effective on samples which had been air dried, in an attempt to replicate forensic casework. This paper specifically

presents the results of the SAPSWash method repeated with human semen. It was considered imperative to demonstrate that it was possible to both recover intact spermatozoa that could be visualised by microscope to confirm the presence of semen and to generate a DNA profile from any recovered spermatozoa. The results of the DNA analysis, undertaken by a UK forensic service provider using ESI17, are presented.

The Office of National Statistics [9] reported that in the twelve months up to March 2017, "an estimated 3.1% of women (510,000) and 0.8% of men (138,000) aged 16 to 59 experienced sexual assault" and these figures do not include offences against minors or the elderly. The SAPSWash method could assist the investigation of sexual offences against all age ranges of potential complainants. Of those who reported sexual offences, "nearly two-thirds (63%) of victims suffered mental or emotional problems as a result, while around half (53%) reported having problems trusting people or having difficulty in other relationships. 1 in 10 victims attempted suicide as a result". The impact of this improved method for the detection of semen cannot be underestimated.

2 Methods and materials

As this work was undertaken as part of a postgraduate study rather than in a forensic service provider laboratory, access to suitable samples for analysis was not straightforward. The method development work was undertaken using a source of boar semen. The final testing of the SAPSWash method was undertaken using a screened, human semen sample from a donor, identified via a private fertility clinic. As it was important to confirm the source of any spermatozoa recovered to the donor, a reference buccal scrape from the human semen donor was obtained.

2.1 Receipt and storage of boar semen samples

Boar semen samples were sourced (JSR Genetics Ltd., Driffield, England). The samples were supplied in 75 mL raw liquid semen packs which were separated into 10 mL aliquots and stored frozen at -4 ^{o}C to reduce the freeze thawing effects known to have an adverse effect on semen [8]. The concentration of each aliquot was calculated on

thawing for each tube before use to take into account any sample deterioration over time. A set of serial dilutions was made from each selected aliquot of boar semen in order to accurately estimate the concentration of spermatozoa in each aliquot. It was established that a 1/100 dilution reliably provided the most concentrated dilution to permit the visualisation and counting of spermatozoa on a microscope slide.

2.2 Receipt and storage of human semen samples

Human semen samples provided by donors to the Wessex Fertility Clinic had been treated with Sperm Freeze (FertiPro, Beernem, Belgium) cryo-protectant to preserve them for fertility purposes. The preserved samples were stored in 500 µL sterile straws with cotton plugs (Cryo Bio System, Saint Ouen Sur Iton, France) and initially slowly frozen in liquid nitrogen vapour, before being fully frozen in liquid nitrogen at -20^{o}C. The samples provided for use in this work had been prepared as described above and frozen only once since their receipt at the fertility clinic. Once donor permission had been obtained and the clinic were informed that a sample was required for use, it was defrosted into Nunc IVF 11 mL Centrifuge Tubes (Thermo Fisher Scientific, Roskilde, Denmark), centrifuged at 1400 rpm for 5 minutes and treated with Quinn's Sperm Washing Medium (Sage In-Vitro Fertilization, Trumbull, Connecticut, USA) to remove the cryo-protectant before temporary storage in a Repromed Round Bottom Tube 5 mL (Hunter Scientific, Saffron Walden, UK). The cleaned semen samples were supplied for use in 500 µL sterile straws with cotton plugs and transferred for storage in a freezer at -4^{o}C. The sample was provided with a known concentration of 65 million spermatozoa per mL. Given that this was approximately five times the concentration of the most concentrated boar semen sample aliquot used in method development, then the human sample was diluted with molecular biology grade (MBG) water in the ratio of 1:4 in a 20 mL sterile universal tube. The human sample had been obtained and previously stored for fertility purposes, rather than just frozen at -4^{o}C as the boar semen sample had been during method development.

2.3 Preparation of test substrate samples from sanitary towels

Commercially available (Tesco Ultra) sanitary towels (Tesco, Welwyn Garden City, UK) were selected to prepare the test substrate samples. A grid of 1 cm x 1 cm squares

was drawn on the reverse of the plastic backing wrapper and these were cut using scissors (Figure 2.1). Each 1 cm x 1 cm square had 300 µL of neat semen added, which was pipetted directly through the upper, non-woven layer. As the objective of this work was to establish whether semen could be recovered specifically from the SAP, the upper, non-woven layer of fabric from each 1 cm x 1 cm piece was removed and retained in a 1.5 mL Eppendorf tube (Fisher Scientific, Leicestershire, UK) for later examination if required. No further contact or pressure was applied to replicate normal wear, as this could not feasibly be done uniformly and the upper layer was removed approximately one minute after the semen was applied to the test substrates. Similarly, as the plastic wrapper layer would be unlikely to be recovered as part of a worn, casework exhibit, this layer was removed and discarded. The prepared substrate squares were each transferred to individual 1.5 mL Eppendorf tubes.

The test substrate samples for this work were uniquely labelled so they could be easily distinguished for clarity of sample handling at the forensic laboratory, who were to be processing the DNA analysis. A set of three samples were prepared, immediately frozen at -4^{o}C overnight and defrosted approximately 24 hours later to undertake the extraction process. A further set of three samples were prepared at the same time, but left to air dry overnight, then placed in their respective Eppendorf tubes before the extraction protocol was performed. These air-dried samples were not frozen before the extraction process was performed. The frozen test substrate samples were labelled HF-A, HF-B and HF-C and the air-dried samples were labelled HAD-A, HAD-B and HAD-C, with the H denoting “human”.

Figure 2.1: Tesco Ultra sanitary towel and 1 cm wide sections from which were cut 1 cm x 1 cm test substrate samples.

2.4 SAP Sperm Wash (SAPSWash) Extraction method

The 1 cm x 1 cm test substrate seeded with 300 µL of human semen had been placed into a DNA free a 1.5 mL Eppendorf tube. The substrate was incubated at room temperature for one hour in 1 mL of 0.5M $CaCl_2$ with the lids sealed, taking care to ensure the substrate was fully submerged in the solution. After one hour, the $CaCl_2$ liquid was removed to a new 1.5 mL Eppendorf tube and the test substrate was carefully transferred to a Costar spin basket (Corning) and retained in a separate 1.5 mL Eppendorf tube. The transferred liquid was then spun in a centrifuge (Eppendorf, Centrifuge 5430 R) for one minute at 15000 rcf. The supernatant was removed and discarded, taking care not to dislodge the cell pellet – which may be distributed up the side of the Eppendorf tube. It should also be noted that the supernatant was prone to frothing at this stage. After this step any recovered spermatozoa will be contained in the cell pellet, however, it was noted that if the breakdown of the SAP gel was incomplete it can appear as gel like residue. The pellet was then re-suspended twice more in a further 1 mL of 0.5M $CaCl_2$ and then centrifuged again each time for one

minute at 15000 rcf. The supernatant was again carefully removed and discarded and followed by a final re-suspension in 1 mL of microbiological grade (MBG) water (Fisher Scientific), as a wash step followed by a final centrifugation for one minute at 15000 rcf. This supernatant was then removed and discarded and the resultant cell pellet was re-suspended in 100 µL dH_2O to facilitate quantitation if required. A schematic diagram depicting this SAPSWash method is provided in Figure 2.2.

2.5 Variables considered in method development

In method development the following variations were also considered:

- salt solution concentration
- the volume of salt solution used for extraction
- recovery of extraction product from the incubation liquid both separately and in combination with that recovered from the soaked test substrate (named as "Separate Multi-Wash" and "Combined Multi-Wash" methods respectively)
- varied numbers of salt solution washes post incubation
- frozen and air-dried test substrates

The incubation time and temperature were not varied in this study.

2.6 Quantitation in method development

A series of 1 µL aliquots of the resultant cell suspension were placed onto the prepared microscope slide on a calibrated hotplate (PC-620D, Corning) at 70^{o}C. It was preferable to spot more than one x 1 µL spots to take into account the likely stochastic variation on the sampling. Once dry (after approximately 10 minutes), the spots were stained with Haemotoxylin and Eosin, taking care not to dislodge the dried cell spot, then covered with a glass coverslip and searched sequentially for the presence of spermatozoa using a compound microscope at x 400 magnification. The total number of observed spermatozoa in the 1 µL aliquot were counted and recorded to facilitate the calculation of the percentage recovery of spermatozoa.

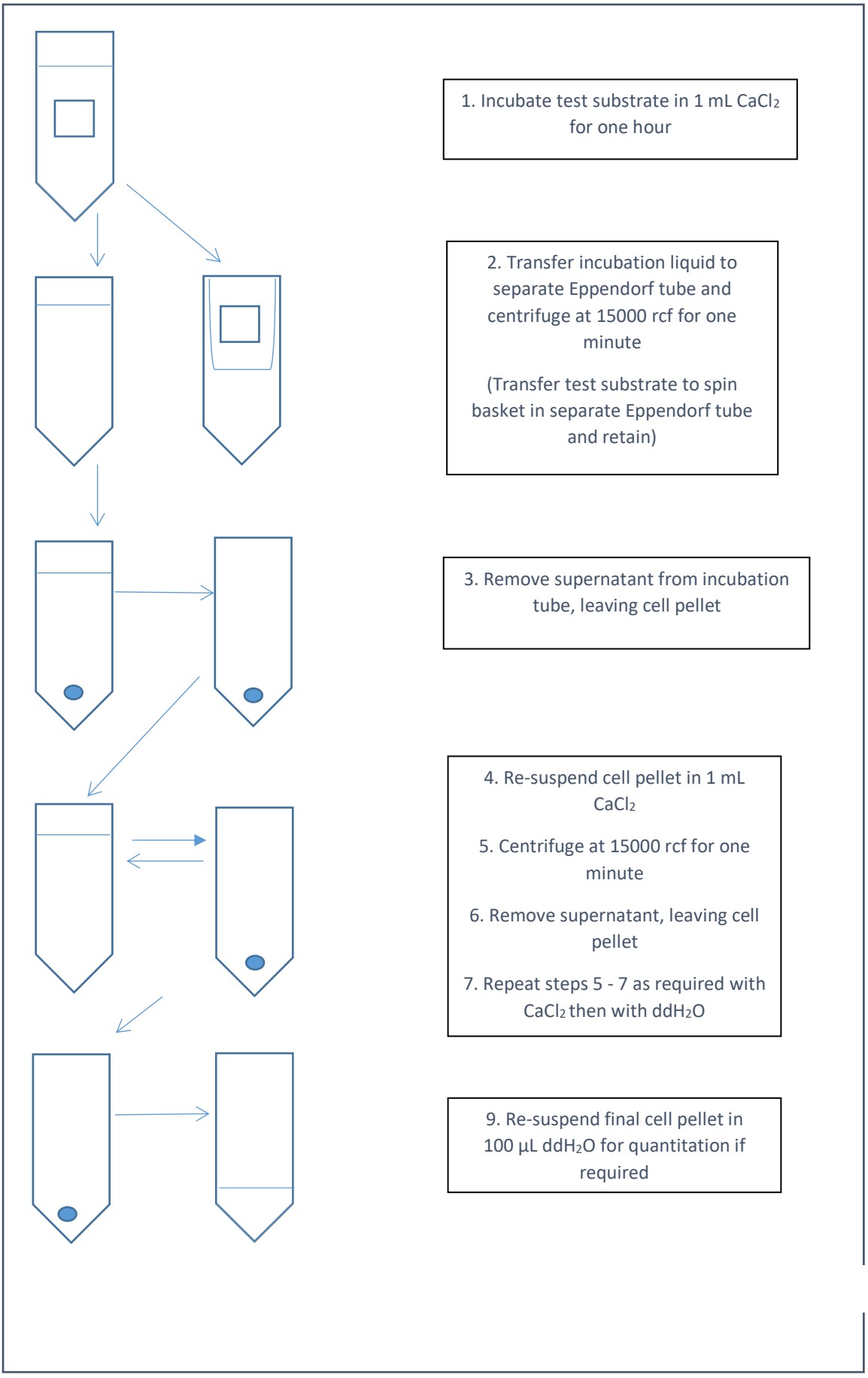


Figure 2.2: Super Absorbent Polymer Sperm Wash (SAPSWash) method

2.7 Method for water extraction of non-woven upper layer of sanitary towel test substrates for recovery of spermatozoa

The individual non-woven upper layer samples had been placed in 1.5 mL Eppendorf tubes at the test substrate preparation stage. These were immersed in 1 mL ddH_2O and vortexed for approximately 20 seconds. The non-woven fabric samples were left in place in the tube and a series of 1 µL aliquots were removed and spotted onto microscope slides, dried, stained and searched as previously described.

2.8 The effect of the SAP on spermatozoa detection limits

The upper surfaces of another set of substrates prepared in the same decreasing volumes were extracted as described in section 2.7 and the findings reported in Table 3.3, to identify whether there was a minimum semen volume beyond which no spermatozoa remained on the upper surface.

The SAPSWash method was used to measure the detection limits of the spermatozoa recovery protocol. The method was applied in triplicate to test substrate samples seeded with decreasing volumes of boar semen (300, 200, 100 and 50 µL) and the results obtained from a series of 1 µL aliquots were presented (Table 3.4).

2.9 DNA analysis

The DNA analysis undertaken on the human semen samples was kindly provided by Key Forensic Services Ltd (Coventry, UK), using the following method. Manual DNA extraction was carried out using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), with an addition of dithiothreitol (DTT) to extract the DNA from the spermatozoa, giving a final extract volume of 50 µl. Each sample was quantified, in duplicate, using the Plexor® HY real-time PCR quantification kit (Promega, Maddison, Wisconsin, USA). Allele amplification was carried out using PowerPlex® ESI17 Fast PCR kit (Promega, Maddison, Wisconsin, USA) and electrophoretic separation carried out on the Life Technologies™ 3130xl (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

There was no attempt to separate the spermatozoa found from any cellular material which may have been present.

The DNA Profile analysis was carried out using GeneMapper ID-X v1.3 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Prior to the DNA analysis it was not known whether the SAP gel would inhibit the reactions. Any samples which provided incomplete DNA profiles were reworked using a Microcon® Centrifugal Filter (Merch Millipore, Burlington, Massachusetts, USA), clean up step with a final volume of 20 µl. Such samples were reprocessed through the quantification, PCR and Capillary Electrophoresis stages of the DNA analysis process.

2.9 Statistical analysis

The Analysis of Variance to establish whether there was any statistical significance between the salt solution concentrations was performed using Microsoft Excel. The remaining statistical analysis on the spermatozoa recovery data was carried out using Minitab 18 [10]. The data sets obtained from each of the experimental methods did not exhibit normal distributions therefore, comparisons were examined using the non-parametric tests. Specifically, the Friedman test was used for related experimental data (where subsequent $CaCl_2$ washes were performed on samples) and the Kruskal-Wallis test was used for non-related experimental data (between different methods) [11,12].

3 Results

3.1 Statistical analysis in method development

Despite a visible increase in size of cell pellet with increased concentrations of $CaCl_2$, no statistical difference in detection level of spermatozoa was observed (*p*-value = 0.602).

The different parameters within the Separate and Combined Multi-Wash methods described in section 2.5 were compared. The *p*-values for the relevant comparisons were all greater than 0.05, indicating that there were no significant differences seen between two and three $CaCl_2$ washes (Table 3.1), or between the relative spermatozoa harvest from recovering the cell pellet from the incubation step separately from that obtained from the soaked test substrate or combining the two (Table 3.2). Critically, no significant difference in spermatozoa recovery was seen between frozen and air-dried test substrates (Table 3.3) where again the *p*-values were all greater than 0.05.

Repeat	Frozen 2 wash	Frozen 3 wash	Air-dried 2 wash	Air-dried 3 wash
P2	0.827	n/a	n/a	n/a
P3	n/a	0.827	n/a	n/a
B2	0.513	n/a	n/a	n/a
B3	n/a	0.827	n/a	n/a

Table 3.1: A summary of the *p*-values obtained using Minitab to perform the Kruskal-Wallis test for unrelated samples on the spermatozoa recovery data obtained from the Combined Multi-Wash and Separate Multi-Wash methods. These protocols have examined the effectiveness of additional repeated $CaCl_2$ washes on the desorption of spermatozoa from the SAP and whether there was any added value in combining the extracts from both the initial incubation liquid and the soaked test substrate. P = cell pellet only; B = substrate in basket filter.

Repeat	Pellet 2 wash	Pellet 3 wash	Basket 2 wash	Basket 3 wash
P2	n/a			
P3	0.083	n/a		
B2	0.083	n/a	n/a	
B3	n/a	0.083	0.083	n/a

Table 3.2: A summary of the *p*-values obtained using Minitab to perform the Friedman Test for related samples on the spermatozoa recovery data obtained from the separate pellet and basket extractions using both two and three $CaCl_2$ washes. P = cell pellet only; B = substrate in basket filter.

	Frozen 2 wash	Frozen 3 wash	Air-dried 2 wash	Air-dried 3 wash
F2	n/a			
F3	0.083	n/a		
AD2	0.127	n/a	n/a	
AD3	n/a	0.507	0.083	n/a

Table 3.3: A table summarising the *p*-values obtained using Minitab to perform the Friedman Test for related samples and the Kruskal-Wallis test for unrelated samples on the spermatozoa recovery data obtained from the Combined Multi-Wash method using both two and three $CaCl_2$ washes. F = Frozen samples; AD = Air-dried samples.

3.2 Preparation of test substrate samples

Given the similarities between the physical properties of boar and human semen, it was not anticipated that the SAPSWash method would be hindered by the use of human semen. However, as the human semen sample provided was approximately five times the concentration of the boar semen used for the developmental experiments, it was diluted with ddH_2O in order to replicate as far as possible the conditions of the rest of the study. As there had been some challenges throughout

method development with maintaining the boar semen sample homogeneity, there was some concern that diluting the stock semen sample may have had an effect, but it was demonstrated microscopically that each of the six tested samples, made with human semen, had spermatozoa which had been successfully released from the SAP within the test substrates present before commencing the DNA analysis.

3.3 Spermatozoa recovered from retained upper surfaces of test substrates

The water extraction method described in section 2.7 was used to investigate the limit beyond which all semen deposited was apparently fully absorbed by the SAP and not remaining on the upper surface of the ultra-sanitary towel test substrates (Table 3.4)

Neat semen volume:	**300 µL**	**200 µL**	**100 µL**	**50 µL**
A	148	0	0	0
B	192	0	0	0
C	220	0	0	0

Table 3.4: Measurement of spermatozoa remaining in the upper layer of test substrates. Results of 1000 µL ddH_2O extraction of non-woven fabric layer removed from test substrate samples seeded with 300 µl boar semen on 1 cm x 1 cm cut samples of sanitary towels. The numbers shown indicate the counted numbers of spermatozoa present in 1 µL aliquots taken from the extraction product.

The SAPSWash method was used to measure the detection limits of the spermatozoa recovery protocol. The method was applied in triplicate to test substrate samples seeded with decreasing volumes of boar semen (300, 200, 100 and 50 µL) and the results obtained from a series of 1 µL aliquots were presented (Table 3.5).

3.5 DNA analysis

The researcher's ESI17 DNA profile was provided for comparison purposes, in order to eliminate any sample contamination as this work was not completed in a designated DNA clean laboratory. A reference DNA profile was obtained from the buccal scrapes provided by the donor of the human semen sample (Figure 3.1), also for comparison purposes.

ESI17 DNA STR results were obtained from the samples designated HF-A, HF-B, HF-C, HAD-A, HAD-B and HAD-C. Full profiles indicating a full complement of alleles were obtained from samples HAD-A (Figure 3.2) and HAD-C. Partial profiles, indicating an absence of alleles at some loci were obtained from samples HF-A, HF-B and HAD-B and no result was obtained from sample HF-C. The samples which had produced incomplete, partial DNA profiles or failed results were sent for a further clean-up step using Microcon analysis (described in section 2.10) and these repeated experiments produced complete profiles. The autosomal and Y-STR quantification results from each of the samples were produced in Table 3.6.

	0.5 M 300 µL A	0.5 M 300 µL B	0.5 M 300 µL C	0.5 M 300 µL H_2O
A	36	2	34	1
B	22	5	79	3
C	46	5	35	1
D	22	2	46	0
E	51	1	26	2
F	18	1	36	2

	200 µL A	200 µL B	200 µL C	200 µL H_2O
A	5	2	1	0
B	3	1	1	1
C	2	6	0	0
D	7	2	1	1
E	4	2	2	1
F	13	2	1	0

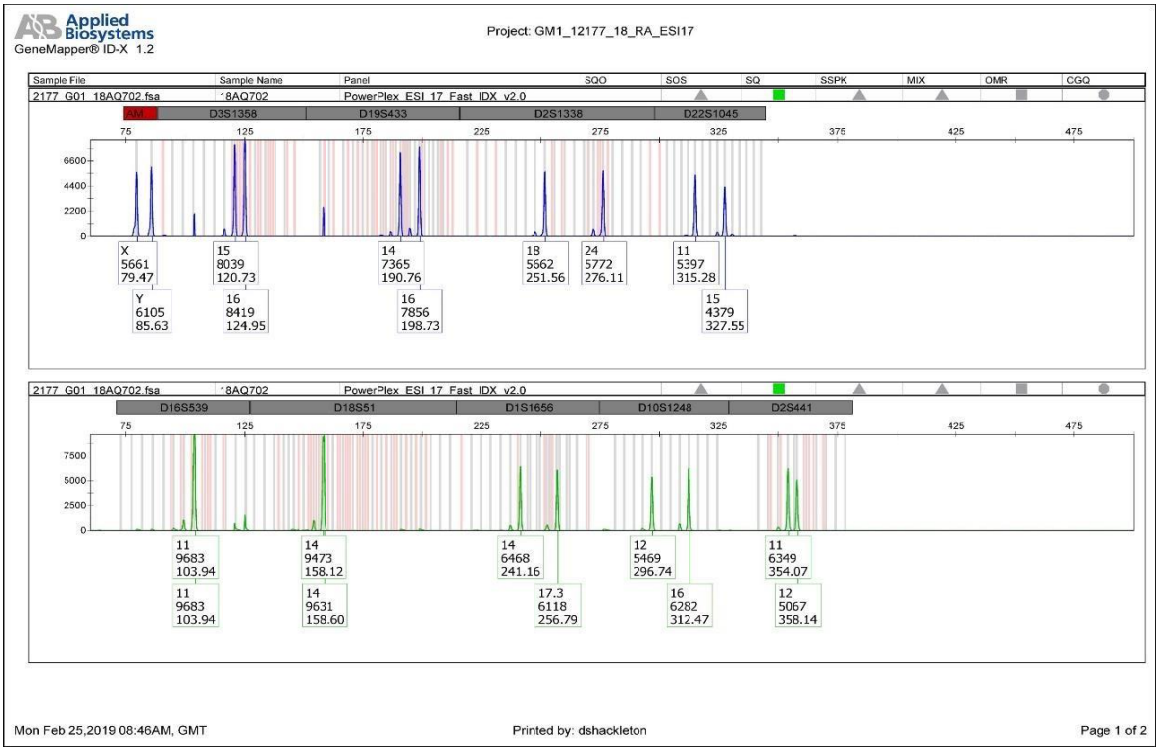
	100 µL A	100 µL B	100 µL C	100 µL H_2O
A	0	0	0	0
B	0	0	0	0
C	0	1	0	0
D	0	0	1	0
E	0	0	0	0
F	0	0	0	0
Averages	0	0.2	0.2	0

	50 µL A	50 µL B	50 µL C	50 µL H_2O
A	0	0	0	0
B	0	0	0	0
C	0	0	0	0
D	0	1	0	0
E	0	0	0	0
F	0	0	0	0

Table 3.5: Results of experiments, in triplicate to assess the detection limits of the SAPSWash method, using two x 1 mL washes with 0.5M $CaCl_2$ only on the test substrate which had been incubated in 1 mL of 0.5 M $CaCl_2$ and without combination of the supernatant from the substrate. The numbers shown indicated the numbers of spermatozoa present in 1 µL aliquots taken from the extraction product and their respective averages.

	Pre-Microcon Quant Score ng/µL	Y Quant Score ng/µL	Microcon Quant Score ng/µL	Y Quant Score ng/µL
HF-A	0.068	0.252	0.093	0.206
HF-B	0.053	0.202	0.079	0.146
HF-C	0.078	0.250	0.084	0.180
HAD-A	0.014	0.047	0.029	0.035
HAD-B	0.058	0.163	0.074	0.134
HAD-C	0.042	0.132	0.074	0.119

Table 3.6: Autosomal and Y-STR DNA quantitation values for all human semen samples extracted using the SAPSWash method. Quantification is measured in nanograms per microlitre (ng/µL)



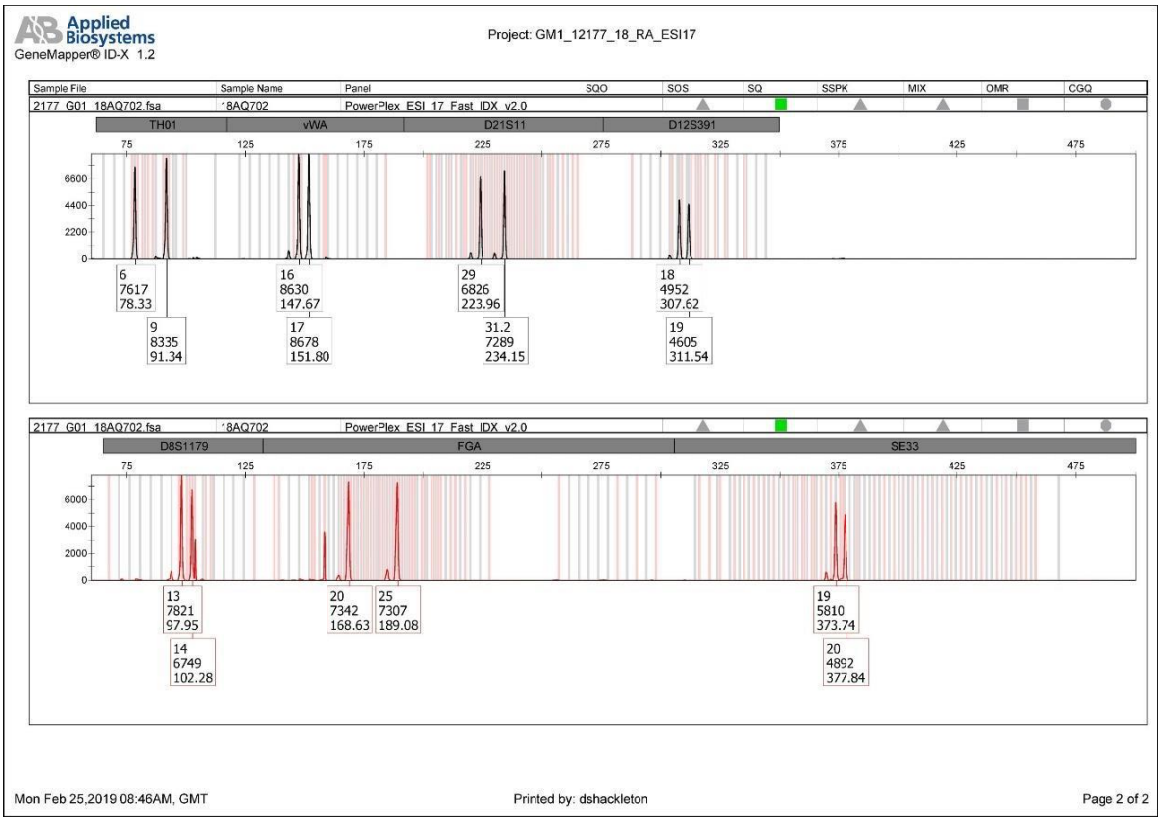


Figure 3.1: ESI17 DNA STR profile obtained from reference buccal scrape provided by the human semen donor.

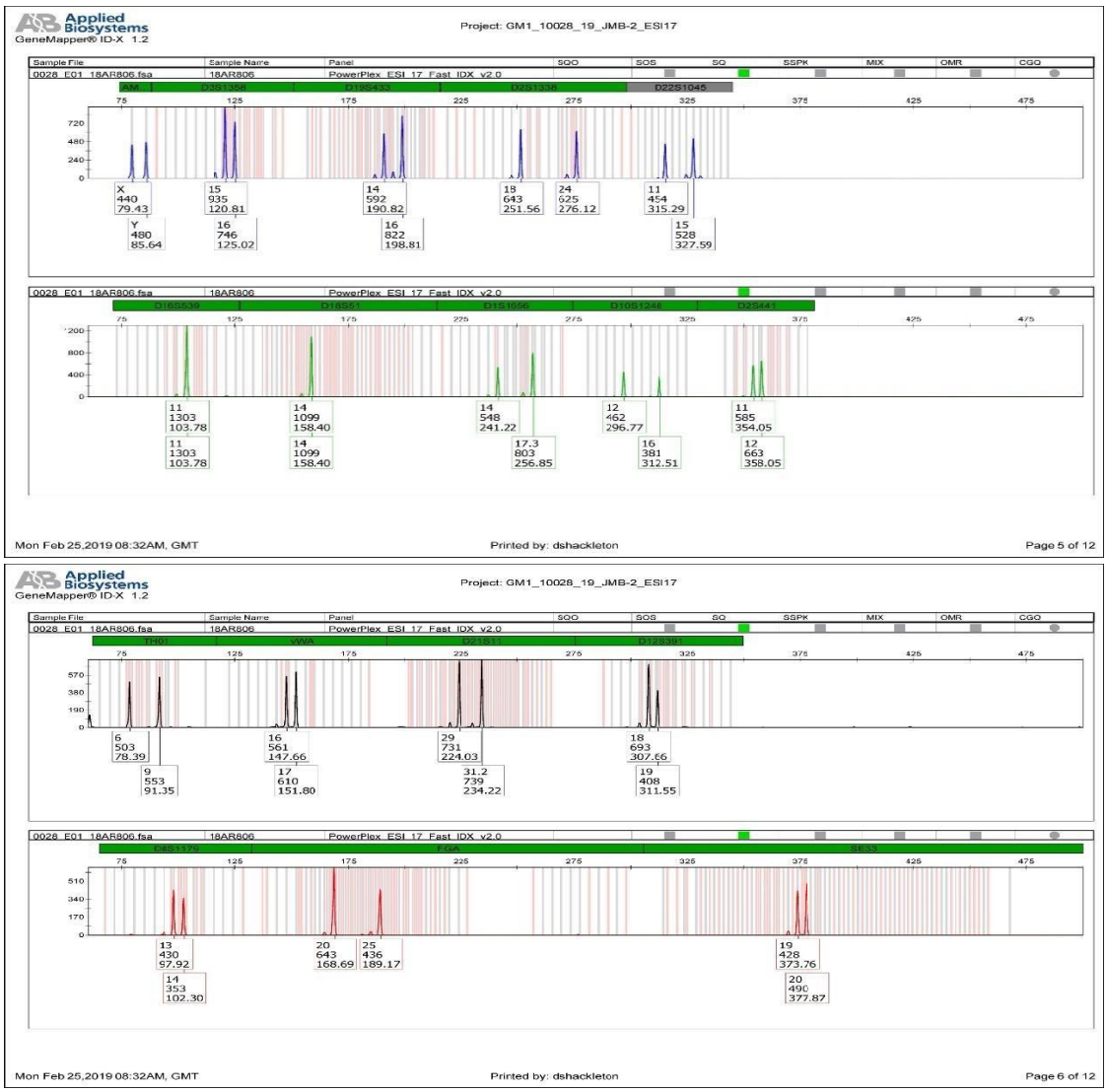


Figure 3.2: ESI17 DNA STR result obtained from extract designated HAD-A, produced using SAPSWash method.

4 Discussion

The objective of this work was to establish whether spermatozoa could be released from the SAPs used in the manufacture of modern sanitary protection products. The results from this study indicate that SAPSWash is a reliable and rapid method for the removal and detection of spermatozoa from SAPs. The yield of the spermatozoa was comparatively poor considering the number of spermatozoa applied to the test substrates but sufficient amounts were recovered to yield a full DNA profile, displaying the full complement of ESI17 alleles matching that of the human semen donor. This finding represents a vast improvement on current methods which cannot recover any semen from the SAP in these types of exhibit substrate.

It was recognised that there are a number of ways this method could be optimised to enable successful adoption by operational forensic science service suppliers and these are discussed here.

4.1 Sample handling

Semen composition varies between donors and has a number of physical properties which make it challenging to manage and interpret. Most of the literature concerned with semen sample handling is related to its use for fertility purposes and stated that the use of a vortex was not recommended to avoid damage to the spermatozoa [13]. It was also widely stated that semen samples agglutinate and spermatozoa within samples tend to clump together [13]. Vortex induced damage, including removal of the spermatozoa tails would clearly affect motility, which would be an obvious issue for fertility purposes. However, motility was not considered an issue for forensic samples so a vortex step was added to the sampling aspects of the procedures in this work in an attempt to maintain the homogeneity of the sample. Since the spermatozoa tails assist with fixing sperm to substrates, their detachment could be considered a benefit in the forensic recovery context. Maintaining homogeneity in neat semen samples was difficult without some attempt at manual mixing and prolonged storage appeared to have an adverse effect on sample concentration. This was demonstrated by the decrease in concentration of samples which had been obtained and frozen at the same time from the same original stock samples.

A number of issues regarding sample to slide adhesion were overcome in the method development stages. Whilst it was possible to obtain an extraction product using salt solutions, attempts to adhere the product to microscope slides for visualisation were hindered, either by insufficient breakdown of the SAP creating a gel like extract which would not dry, or a product was obtained which retained a crystalline property which cracked and washed off the slide once dry. Increasing the number of salt washes removed this issue and the introduction of a final water washing step after the salt solution washes facilitated the slide adhesion, essential for visualisation of the recovered spermatozoa.

Spermatozoa were recovered from the SAP in each of the experimental methods, using a range of salt solution concentrations and at decreased volumes of template semen samples. This indicated that spermatozoa recovery was possible and it was likely that yield could be improved by further method optimisation. Whilst the extraction products delivered spermatozoa clearly free from SAP gel, it was also observed that there were some present with tails stuck within the gel matrix. It was likely that this also had an effect on the apparent stochastic variation within each of the samples when measured for spermatozoa recovery. It was not anticipated that this would have an effect on any subsequent DNA analysis since the sperm heads themselves appeared to be free from the gel and available for DNA extraction. However, it was considered that the gel itself may behave as an inhibitor to the DNA analysis process hence the decision to engage with a forensic service provider to undertake forensic industry standard DNA analysis.

4.2 Method development

Whilst the main objective of this work was to establish whether it was possible to break down the SAP sufficiently to release the spermatozoa from the gel, the efficiency and practicality of the process was considered. Forensic science is increasingly measured on speed and cost effectiveness as well as accuracy and precision, so all proposed new methods should aim to balance these aspects. However, forensic scientists can only base their expectation of findings on alleged case circumstances and will never know in advance categorically what they may find on an exhibit. Therefore, any new method should seek to cover a range of outcomes where possible. This method delivered an

effective, efficient and reproducible process for recovering spermatozoa from SAPs using inexpensive and widely available consumables, compatible with existing forensic techniques. The success of the spermatozoa recovery from the human semen samples was tested by attempting industry standard DNA analysis and measuring the quantification values rather than by counting the number of spermatozoa, particularly because of the known non-homogeneity of semen. Any comparison of counted spermatozoa with time since intercourse studies would not be recommended

4.3 DNA analysis

There are a number of known PCR inhibitors encountered in forensic DNA casework (the indigo dye in denim fabric being a common one) and dilution of inhibited samples can permit the generation of DNA profiles from them since this has the added effect of diluting the inhibitor. Whilst more recent advances in DNA chemistry has minimised the effects of inhibitors, the developed experimental methods had been shown to not fully breakdown the SAP gel, therefore it was known that at least some was likely to be present in each of the extracts. It was important to demonstrate whether any semen recovered from the SAP was suitable for DNA analysis since, in terms of forensic investigation, the recovery of the semen from the SAP would be of limited use if a DNA profile for comparison purposes could not be generated from it.

Of the six samples tested, inhibition was seen in all three of the samples which had been previously frozen and only one of those which had been air dried. The inhibition was seen by the absence of alleles at one or more loci and could be distinguished from sample degradation by the absences not being necessarily only at the high molecular weight loci. The Microcon test, commonly used in forensic DNA analysis as a clean-up step for samples which have not produced a full profile at first attempt, was applied to each of the samples which gave inhibited DNA results at first attempt. Each of these samples then produced full DNA profiles.

These results illustrated two clear benefits of this study. It was encouraging to obtain full DNA profiles from all of the human semen extract samples using the routine DNA analysis techniques which have been standard and robust in the UK forensic laboratories for a number of years. This implied that the extraction method could be adapted for implementation in forensic casework use with only limited amendment to

existing techniques for DNA analysis. Furthermore, the DNA analysis was shown to be successful on the samples which had been left to air dry overnight, a process which had been included to attempt to replicate a live casework exhibit.

Every effort had been made to avoid the potential for sample contamination, including wearing appropriate personal protection equipment, utilising DNA free consumables and working alone in the laboratory. Given that the test substrate preparation and the preparatory SAP extraction work had been done in a non-designated DNA clean laboratory, it was also reassuring that there was no evidence of contamination present within the DNA results obtained, either from the researcher or other unknown sources, particularly as there was no attempt to separate the sample into sperm and cellular fractions prior to DNA extraction.

4.4 Key Findings

4.4.1 Sample handling - semen

It was noted that the concentration of the stored boar semen during method development deteriorated over time with prolonged storage in a freezer at -4^{o}C and no use of no cryo-protectant. It was clear from the literature that semen samples stored for human fertility purposes were stored under liquid nitrogen and with a cryo-protectant. The human semen sample provided had been stored in this way and no adverse effects were observed either regarding the ability to recover the semen from the SAP or achieving a full DNA profile. It would therefore be recommended that any further work considered using human semen was done using either fresh samples available for immediate use, or using the long term storage methods recommended for samples taken for fertility purposes.

Semen sample viscosity can be highly variable between samples and between donors so appropriate methods of sample handling should be employed to ensure, as far as possible, the homogeneity of the samples when attempting to quantify recovery. It is not sufficient to simply shake the vessel containing a recently thawed semen sample to equilibrate it. The shaking action, or even the use of a vortex, can damage the spermatozoa and detach any tails present so it is preferable to use a less destructive

method to homogenise any samples. Convention suggested that repeated drawing in and out of the sample through a large bore pipette should both homogenise the sample and improve its viscosity for ease of sample handling. Maintaining homogeneity in neat semen samples was difficult and prolonged storage appeared to have an effect on sample concentration. Preparation of diluted samples introduced a further level of variability as it proved difficult to achieve sample homogeneity when diluting with water to try to replicate samples of reduced concentration.

4.4.2 Comparison with forensic casework

This work has demonstrated that there was no significant difference in the recovery of spermatozoa between test substrate batches which had been prepared freshly, from the same semen sample, at the same time, with one batch being immediately frozen and another being allowed to air dry overnight. Clearly, it remains preferable that any exhibits are recovered and stored following crime scene examination guidelines as swiftly as possible after an alleged offence has occurred.

However, it has been established that it is now preferable to extract items for semen using buffer solutions, such as Mo Lite or Mo Classic as described by Hulme *et al*., (2012) [2], rather than water. There can be a fine balance between detecting a body fluid and obtaining a DNA profile which can be attributed to it and so current practice is to extract body fluids into controlled pH environments compatible with any subsequent DNA analysis. This is similarly reflected in the approaches taken by Camarena *et al,* (2017) [7] and Gregório *et al* (2019) [8].

In conversation with G. Davidson, (October 2018) it was discussed that with low levels of spermatozoa it is more common that DNA quantification is measured to assess likelihood of successful generation of a DNA profile rather than an arbitrary measure of the number of spermatozoa present. It can also be useful in forensic casework to test recovered samples for the presence of male DNA using Y-STR analysis to specifically target male DNA. Whilst this is possible, Y-STR testing targets all male DNA and as such is not specific to DNA from spermatozoa. The relevance of detecting male DNA would be case specific and may not be appropriate if the presence or absence of spermatozoa was critical in a particular sexual offence case.

4.4.3 DNA analysis

Of the six samples tested using the SAPSWash method on human semen samples, only two gave full profiles at the first attempt. Both of these samples were those which had been air dried before extraction so it was encouraging from the perspective of achieving DNA profiles in a live forensic casework environment. The remaining four samples also produced full DNA profiles but only after implementation of the Microcon clean-up step. This additional treatment does form part of standard forensic DNA analysis given the knowledge that some substrates present inhibition challenges. It could be considered that it is not possible to determine whether these samples were initially unsuccessful due to the SAP gel being an inhibitor, or because three of the four had been stored frozen before they were exposed to the extraction process, or indeed a combination of both. However, the full profile DNA results obtained indicated that neither of these options represented a significant challenge.

It is worthy of note that casework samples may reasonably be expected to be mixed with other body fluids. Such samples would be typically subjected to routine techniques to separate spermatozoa from any cellular material in order to both remove any exhibit donor DNA and to reliably attribute any male DNA profile obtained specifically to semen. Such pre-separation may, arguably negate the need for any Microcon treatment at the DNA analysis stage.

4.5 Recommendations for future work

In considering the findings of the work, there were several recommendations for further experiments in order to consider this method for implementation into standard forensic laboratory operating procedures. The ultimate aim would be for the introduction of an efficient, cost effective and successful method of releasing spermatozoa from SAPs to further forensic investigation of sexual offences.

1. Some current casework methods used to extract semen from intimate swab or fabric exhibits use buffer solutions rather than water. These have come to be considered to replicate a more natural environment for cellular material and were thought to be less harmful than ddH_2O which can swell and lyse the cells.

Buffer solutions could be investigated as a replacement for the ddH_2O used in all of the methods described in this work.

2. Some current casework spermatozoa recovery methods include cell lysis steps and sample clean-up steps in the initial spermatozoa extraction method prior to any DNA analysis. Any attempt to integrate the SAPSWash method into current casework could consider both of these aspects in an attempt to further optimise an overall method before casework validation and implementation into any laboratory standard operating procedures.

3. This work showed no significant differences in spermatozoa recovery between two and three additional salt solution washes. However, the third wash undertaken during method development for both the Separate and Combined Multi Wash methods, was only done after the first two washes and a water wash. It may be considered useful to compare whether three salt washes followed by a final water wash delivered improved SAP breakdown. It may also be useful to consider whether soaking the substrate for longer in a larger vessel or with agitation and / or increased temperature (in a water bath) may eradicate the need for the additional washes altogether since all of this experimentation was completed at ambient laboratory temperature, whereas many current extraction techniques now include an incubation step.

4. This work has considered only test substrates made from excised sections of Ultra sanitary towels. It is recognised that the range of SAP containing products includes products which are much bulkier than those used for this work. It would therefore be recommended that the methods are scaled up to accommodate this by sourcing alternative vessels for the incubation and centrifugation of the samples. Method protocols could be developed to recommend specific volumes of salt solution be used with particular sizes of excised, SAP containing material and examining the effects of bulk extraction versus combining several smaller samples

5. This work looked solely at the recovery of spermatozoa from the SAP and did not address the use of any of the presumptive tests available to evaluate the presence of seminal fluid prior to the identification of spermatozoa or in the absence of spermatozoa as the confirmatory test. While the sanitary protection products would normally be chemically screened prior to sampling to assist in identifying an area for further sampling, it may be considered useful to apply a presumptive test to the SAP beads within the absorbent pulp layer of the sanitary protection products or to test the first resultant supernatant from extraction method to establish whether seminal fluid may be present before completing the full method protocol. This step could be built into any proposed standard operating procedure to inform any user about the relative usefulness of the extraction process if it could be shown that the chosen presumptive test provided additional information. This would need to be considered within the already known reliability parameters of any such tests.

6. It is acknowledged that this method has been developed and shown to work on clean, laboratory prepared samples, wholly uncontaminated with other body fluids. Although not always, such similar forensic casework exhibits would be considered likely to be contaminated with other body fluids and waste material of the item wearer. It is essential to examine whether the methods translate to soiled items, either sourced appropriately or simulated in the laboratory.

Conclusion

The successful retention of liquids in sanitary protection products has been a problem for forensic scientists worldwide since SAPs were first used in nappies and has become more widespread since their incorporation in products for adult use.

The SAP Sperm Wash (SAPSWash) method presented has been developed and shown to deliver full DNA profiles from spermatozoa recovered directly from the SAP from samples. The method has been shown to be effective on samples which are considered to contain significantly lower template DNA than may be expected from a full average ejaculate.

Since SAPs have been found to be present in nappies, sanitary towels and incontinence pads, the successful resolution of the inability to recover semen from the SAP layer could have a significant impact on the investigation of sexual offences across a wide range of potential complainants and in particular those who may be considered more especially particularly vulnerable, such as infants or the elderly.

The SAPSWash method has delivered an efficient, cost effective process which could be relatively easily implemented into current, mainstream forensic laboratory practices, using standard, inexpensive consumables. The method was demonstrably effective in producing evidential quality DNA profiles, therefore the implications for this work are wide reaching and they could deliver immediate impact across a wide range of case types. Whilst it is not known how many cases include SAP containing exhibits, it is known that forensic scientists cannot currently recover semen from the SAP layer so this work could significantly increase the successful examination of exhibits of this nature. It is clear that this method has presented a solution not only to the examination of nappies for semen, but, also the examination of sanitary towels and incontinence pads. Therefore, the SAPSWash method could assist the investigation of sexual offences against all age ranges of potential complainants and the impact of this improved method for the detection of semen cannot be underestimated.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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