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The influence of substrate on DNA transfer and extraction efficiency

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ABSTRACT

The circumstances surrounding deposition of DNA profiles are increasingly becoming an issue in court proceedings, especially whether or not the deposit was made by primary transfer. In order to improve the currently problematic evaluation of transfer scenarios in court proceedings, we examined the influence a variety of nine substrate types (six varieties of fabric, plywood, tarpaulin, and plastic sheets) has on DNA transfer involving blood. DNA transfer percentages were significantly higher ($p = 0.03$) when the primary substrate was of non-porous material (such as tarpaulin, plastic or, to a lesser degree, wood) and the secondary substrate porous (such as fabrics). These findings on transfer percentages confirm the results of previous studies. Fabric composition was also shown to have a significant ($p = 0.03$) effect on DNA transfer; when experiments were performed with friction from a variety of fabrics to a specific weave of cotton, transfer percentages ranged from 4% (flannelette) to 94% (acetate).

The propensity for the same nine substrates to impact upon the efficiency of DNA extraction procedures was also examined. Significant ($p = 0.03$) differences were found among the extraction efficiencies from different materials. When 15 μL of blood was deposited on each of the substrates, the lowest quantity of DNA was extracted from plastic (20 ng) and the highest quantities extracted from calico and flannelette (650 ng). Significant ($p < 0.05$) differences also exist among the DNA extraction yield from different initial blood volumes from all substrates. Also, significantly greater ($p < 0.05$) loss of DNA was seen during concentration of extracts with higher compared to lower initial quantities of DNA. These findings suggest that the efficiency of extraction and concentration impacts upon the final amount of DNA available for analysis and that consideration of these effects should not be ignored. The application of correction factors to adjust for any variation among extraction and concentration efficiencies among substrates is proposed.

1. Introduction

Secondary transfer of DNA can have an important bearing on criminal investigations with its potential to cast doubt over the origin, and, as a consequence, the validity of DNA evidence present at a crime scene [1–13]. This potential has led to requests during court proceedings for assessment of the likelihood of DNA transfer under certain proposed situations [14–17]. Such assessments require an understanding of the variables which can affect transfer. However, to date, only limited research in this area has been published [8,9].

Recent reports of DNA transfer [8,9] focussed on transfer associated with three substrates: cotton (representing a soft and porous material), plastic (representing hard and non-porous surfaces) and, to a lesser extent, wool. Significant differences in transfer could be attributed to substrate types; transfer from a non-porous substrate to a non-porous secondary substrate reached a maximum average of 64% whilst transfer from the same substrate to a porous surface attained a maximum average of 100% [8]. There are, however, many other types of substrate which can be present at crime scenes and it is currently unknown how these, and their characteristics, such as porosity, composition and density, may influence DNA transfer. Other factors which may influence the assessment of DNA transfer, such as differences in the loss of DNA during extraction and concentration processes, were also not examined in the earlier studies [8,9].

A substrate class commonly associated with DNA evidence is fabric as it forms many items of clothing worn by persons involved in or affected by crime, as well as a variety of common household items. Fabrics consist of natural and/or synthetic fibres woven into a matrix, and there are many different fibre compositions of varying weave matrices in common use [18]. It is impractical to analyse all fabrics that could be found at a crime scene. We

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investigated undyed fabrics comprising common fibres, such as cotton, polyester and acetate, woven to form a common, simple weave type known as a plain weave. Flannelette and twill woven cotton were included in order to compare and contrast the effects of more complex weave types on transfer. The set of fabrics we chose allowed examination of the effect of common fibre types, and, to a limited extent, weave types, on transfer.

The substrate on which biological material is deposited may have an effect on the efficiency of DNA extraction. In any controlled study of transfer variables, transfer percentages are calculated by using quantities of DNA collected from two substrates: the primary substrate on which the biological material was deposited, and the secondary substrate to which some of it was transferred. If the efficiencies of the techniques for sampling and/or extraction from one substrate differed from the efficiencies of the same techniques used on a different substrate, the DNA quantities used in calculations of transfer would be inaccurate.

Differences in efficiency may also be present among samples extracted from different initial volumes of blood deposited on the same substrate, leading to greater inaccuracy when calculating transfer between substrates which contain considerably different blood volumes. We hypothesised that different quantities of DNA are obtained from the same volume of biological material deposited on different substrates due to differences in extraction efficiency. Further, that if this is so, then a correction to the values is required.

Although not directly related to substrate characteristics, the efficiency of concentrating trace DNA samples after extraction may vary according to the initial concentration of DNA, and lead to further inaccuracies in the final quantity of DNA determined.

In this study we investigate a range of substrates commonly associated with crime scenes, but hitherto not examined, to determine if, and to what extent, the variables identified by Goray et al. [8,9] affect transfer. The efficiency of the extraction technique used to process DNA from each of these substrates is also examined. Our intention is that the findings will provide greater accuracy in any assessment of the likelihood of specific transfer events which may be considered in criminal investigations and/or court proceedings.

2. Materials and methods

2.1. Substrates

Six fabrics were selected as substrates, each with a different composition of cotton, polyester and/or acetate. Three additional substrates included a slightly porous hardwood variety of plywood and two completely non-porous substrates; polyethylene tarpaulin and plastic transparency. Two of the substrates, cotton drill and plastic transparency (OfficeMax® Australia, catalogue #1219839), were the same as those examined by Goray et al. [8,9], and were included for comparison purposes. Table 1 lists the properties of all 9 substrates examined. As the biological deposit, blood, was in a liquid state, each fabric was placed onto a non-porous plastic transparency to allow any blood which may have seeped through the substrate to be collected. All such substrates were co-extracted with their underlying transparencies.

2.2. Transfer experiments

Transfer experiments were performed involving each of the first seven substrates listed in Table 1. In separate experiments, each of these substrates was used as either primary or secondary substrate, with plastic or cotton acting as the opposing secondary or primary substrate respectively (Fig. 1). Transfer experiments were carried out following the protocols described in Goray et al. [8,9].

15 μL of venous blood collected in EDTA tubes from a donor other than the analyst (allowing assessment of contamination) was deposited onto the primary substrate and transfer was performed either immediately (wet) or after 24 h (dry). Contact between the substrates was for 60 s either with no external influence (passive) or with the aid of a 1 kg weight attached to the secondary substrate and fast movement in all directions (friction). Four replicates of each transfer event were performed (Fig. 1).

2 cm × 2 cm areas, inclusive of the 1 cm × 1 cm sample deposit area of both the primary substrate and its corresponding transfer region on the secondary substrate, were excised separately using a scalpel and cut into smaller squares of approximately 3 mm × 3 mm. These fragments of substrate, combined with plastic underlay where necessary, were then placed into 10 mL tubes for

Table 1
Substrates used in the experiments and their properties.

Substrate	Weave/structure	Composition	Reactive groups[a] along fibres	Comments
Acetate	Plain weave 40 × 30 yarns per cm	100% acetate	Cellulose with most polar groups acetylated	Commonly used in lining dresses and clothing
Polyester	Plain weave 43 × 32 yarns per cm	100% polyester	Mostly hydrophobic semi-polar ester groups	Commonly used in lining garments
Calico	Plain weave 27 × 21 yarns per cm	100% cotton	Cellulose – many polar hydroxide groups	Used for embroidery, shopping bags
Poly/cotton	Plain weave 47 × 30 yarns per cm	65% polyester 35% cotton	Mix of cellulose fibres and polyester	Fabric from a Solutions® brand business shirt
Flannelette	Plain weave treated to have a soft, fibrous finish on both sides 20 × 17 yarns per cm	100% cotton	Cellulose – many polar hydroxide groups	Commonly used in bedsheets and warm garments
Tarpaulin	Plain weave with non-porous moulded coating, green in colour, 42 × 39 yarns per cm	Polyethylene	No polar groups	Stockman Weekender brand, 1000 denier, 100gsm
Plywood	3 mm thick; 3 layers of wood Surface involved in transfer was a longitudinal section of hardwood	Red Lauan, a mahogany-like hardwood	Mostly cellulose and lignin, a complex polymer with many hydrophobic and hydrophilic groups	A common type of wood used for many applications
Plastic	N/A (moulded sheet)	Plastic transparency	No polar groups	OfficeMax® overhead transparencies catalogue #1219839
Cotton Drill	Drill weave (3:1 twill weave) 45 × 22 yarns per cm	100% cotton	Cellulose – many polar hydroxide groups	A strong material used for clothes such as denim and khaki

Note: All fabrics were white unless indicated otherwise to reduce the chance of dye-induced PCR inhibition.
[a] 'Reactive groups' relates to the chemical composition of the fibres from which each substrate is made.

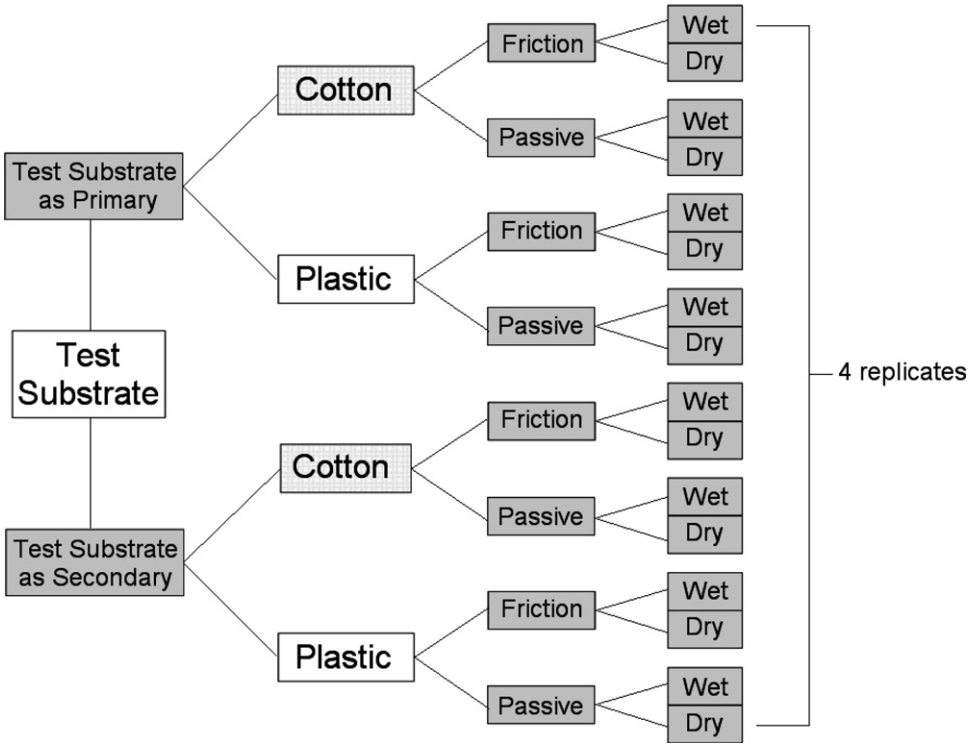

Fig. 1. Schematic diagram of transfer experiments.

extraction. Samples on plywood were swabbed using a wet/dry double swabbing technique, allowed to dry for 24 h, and each swab head was teased off and placed into a 10 mL tube for co-extraction.

Samples were extracted to an end volume of approximately 400 μL using a modified 5% Chelex method [19] (Bio-Rad, USA) previously validated for casework. This extraction process included a phosphate-buffered saline wash step to remove haem followed by a 30 min incubation at 56 °C. Quantification was carried out using Quantifiler® (Applied Biosystems, USA) and an ABI PRISM® 7500 Real-Time PCR system (Applied Biosystems, USA). Amplification was carried out using a 28-cycle PCR with Profiler Plus® (Applied Biosystems, USA), and capillary electrophoresis and profiling performed using an Applied Biosystems 3100 genetic analyser with GeneMapper® ID v3.2. Any sample which tested negative for DNA at the quantification stage was concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore, USA) to an end volume of approximately 50 μL, then re-quantified. All protocols were performed as per manufacturer's recommendations.

Each transfer percentage (T) was calculated using the following formula:

$$T = \frac{D_2}{D_1 + D_2} \times 100 \quad (1)$$

where D_1 is the quantity of DNA extracted from the primary substrate and D_2 is the quantity of DNA extracted from the secondary substrate. Results are presented as the mean and standard deviation of transfer percentages calculated from four replicates of each transfer experiment.

2.3. Controls

DNA profiling was performed on approximately fifty samples, encompassing samples with the highest DNA quantity from each set of transfer experiments, to check for the presence of contaminating alleles. Only the donor's DNA profile was found in each. Negative controls comprising each substrate without biological deposit were also processed. No DNA was detected in any of these negative control samples.

2.4. Extraction efficiency experiments

2.4.1. Experimental design

To test if the efficiencies of DNA extraction were identical among all 9 substrates, a range of volumes of blood (0.1 μL, 0.5 μL, 1 μL, 5 μL, 15 μL and 30 μL) was each deposited onto 4 replicates of 1 cm × 1 cm of each substrate (with underlays as appropriate) as described in Section 2.1. To reduce pipetting error, volumes of blood less than 15 μL were first diluted in TE buffer, i.e. 10 μL of the following dilutions (1 in 100, 1 in 20, 1 in 10, and 1 in 2 respectively) were added. A second set of 4 replicates using 5 μL of blood was examined; however, these used 5 μL of undiluted blood rather than 10 μL of a 1 in 2 dilution. This doubling was performed to assess if there were any differences in DNA retrieval between the diluted 5 μL sample and the undiluted 5 μL sample.

Each piece of substrate with a known quantity of blood deposited onto it was then processed in the same manner as the samples in the transfer experiments (see Section 2.2). All extraction efficiency experiments for one substrate were carried out simultaneously, and among substrates these batches of experiments were carried out within a short time.

2.4.2. Comparison among blood volumes

For each extraction efficiency sample, DNA yield per microlitre was calculated by dividing the amount of extracted DNA by the volume of blood deposited. One-way ANOVA tests (Tukey's multiple comparisons, df = 5) were performed on all calculated DNA yields for each substrate, grouped by blood volume (0.1 μL, 0.5 μL, 1 μL, 5 μL, 15 μL and 30 μL).

2.5. Concentration efficiency

A stock DNA solution (Promega K562) of 27.6 ng/μL (concentration verified using Quantifiler as outlined in Section 2.2) was diluted 1 in 20, 1 in 200 and 1 in 1200 using TE buffer. 400 μL aliquots (to simulate the approximate end extraction volumes generated in transfer experiments) of each dilution were dispensed into 4 pre-labelled Amicon Ultra-4 filter units, and these were concentrated and quantified as outlined in Section 2.2. The amount of DNA present after concentration was calculated by multiplying the final concentration by the end sample volume. The percentage yield was determined by expressing the amount of DNA after concentration as a percentage of the total amount expected in 400 μL of unconcentrated DNA.

2.6. Statistical analysis

All statistical comparisons (unless stated otherwise, as in Section 2.4.2) were performed using the Mann–Whitney *U*-test (significance level $p < 0.05$) using Minitab® v15 (Minitab Inc.).

3. Results

3.1. Transfer percentages for test substrates

Transfer percentages between the various substrate combinations are presented in Figs. 2 and 3 from which a number of general trends can be identified. Firstly, transfer was highest when the primary substrate was non-porous and the secondary substrate porous. Secondly, transfer was significantly higher when friction was applied, and thirdly, transfer was highest for wet rather than dried biological material.

Comparisons of DNA transfer from different fabrics under optimum transfer conditions (i.e. wet deposit transferred with friction to cotton) revealed three significantly different ($p = 0.03$) groups after analysis. The 3 groups in descending order of DNA transferred, were (i) acetate and polyester; (ii) calico and polyester/cotton; and (iii) flannelette (Fig. 2a). Comparing the fabrics as secondary substrates (for transfer of wet blood with friction from plastic) showed that polyester, flannelette and calico had a higher uptake of DNA than polyester/cotton and acetate ($p = 0.03$). When DNA was transferred from cotton to other fabrics, the only significant differences were that more DNA was transferred to polyester than to calico and acetate ($p = 0.03$)

Comparison of the two less porous test substrates, plywood and tarpaulin, with fabrics revealed that both these surfaces, as primary substrates, exhibited greater ($p = 0.03$) transfer than all fabrics (Figs. 2a and 3a) under both passive and friction contact, but that this difference increased with friction. These two surfaces also received less DNA than fabrics when acting as secondary substrates (Fig. 3b). Though the transfer percentage from plastic to plywood was approximately 50%, and to tarpaulin approximately 30%, this difference was insignificant.

3.2. Extraction efficiency

3.2.1. Difference among substrates

At any one volume of blood, extraction efficiency varied among substrates (Fig. 4). This variation in extraction efficiency also differed among blood volumes; when compared pairwise at each blood volume, plastic consistently provided the lowest ($p = 0.03$) amount of extractable DNA, only indistinguishable from 0.1 μL and 30 μL blood deposits on plywood.

3.2.2. Difference among blood volumes

Significant differences in DNA yield per microlitre (amount of DNA divided by blood volume) from different blood volumes on the same substrate are presented in Table 2. Overall, each substrate exhibited varied significant differences in yields at different blood volumes. All substrates exhibited no significant difference ($p < 0.05$) between the yields from 15 μL and 30 μL of blood, however, no other clear pattern emerged among other volumes on each substrate.

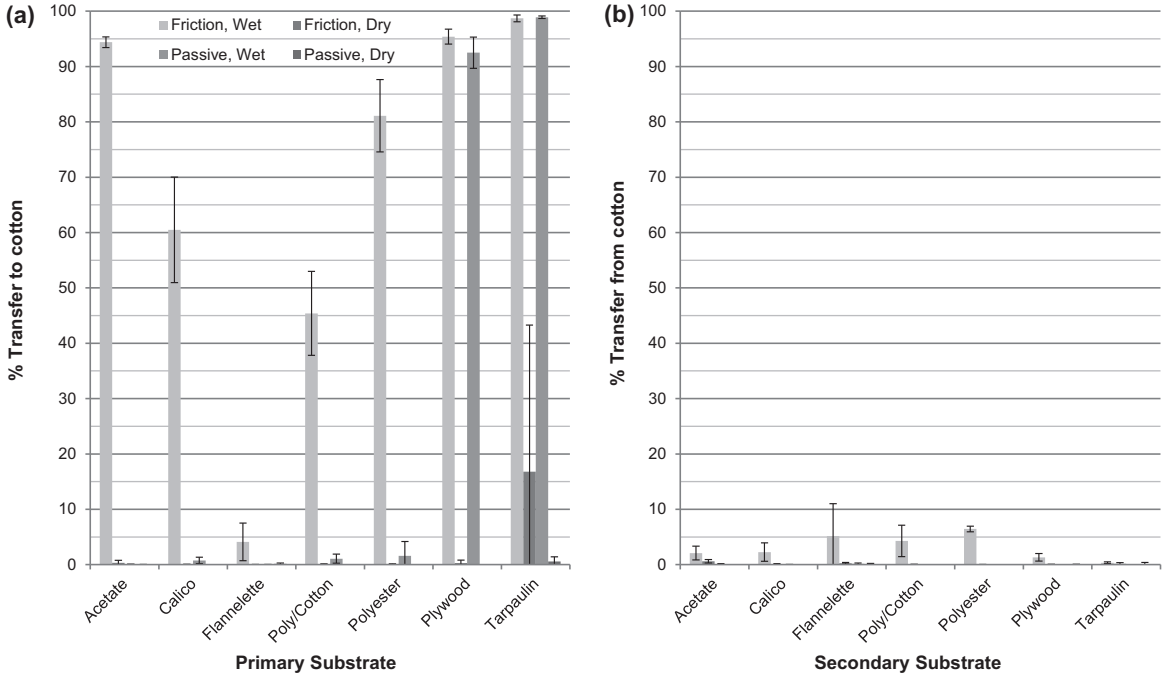

Fig. 2. Transfer percentages for transfer of blood (a) to and (b) from cotton. Each substrate displays transfer percentages (from left to right) for friction transfer of wet blood, friction transfer of dry blood, passive transfer of wet blood and passive transfer of dry blood. Values are presented as percentage of DNA transferred from primary to secondary substrate, error bars display ± 1 standard deviation of transfer percentages.

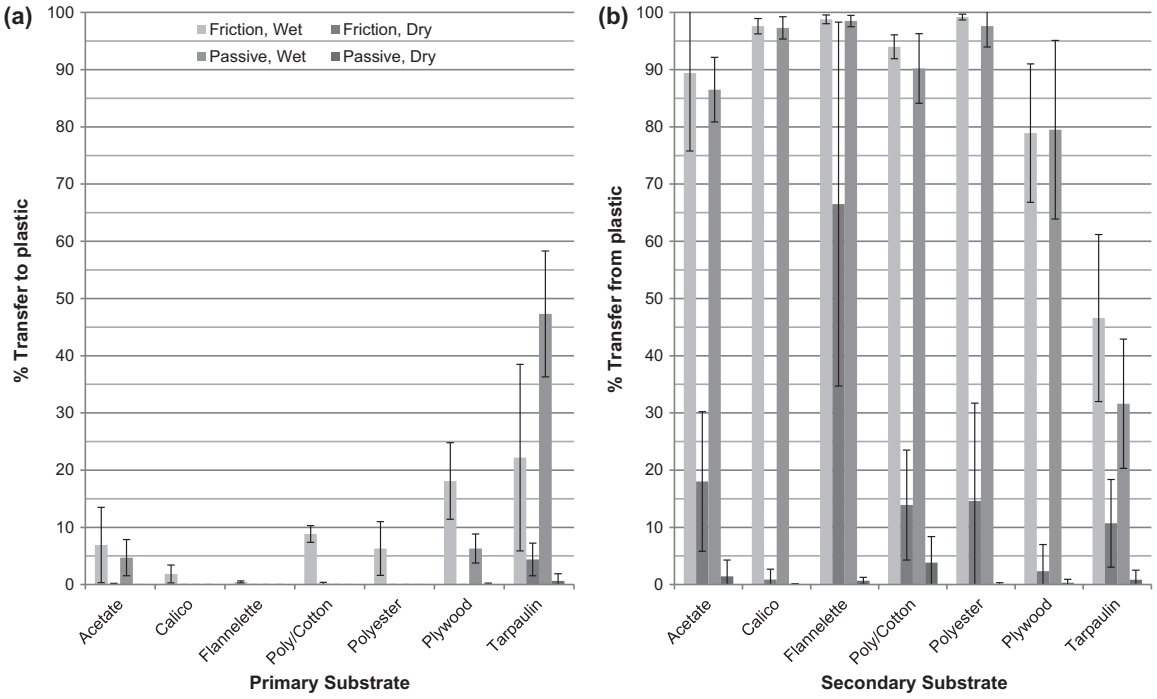

Fig. 3. Transfer percentages for transfer of blood (a) to and (b) from plastic. Each substrate displays transfer percentages (from left to right), for friction transfer of wet blood, friction transfer of dry blood, passive transfer of wet blood and passive transfer of dry blood. Values are presented as percentage of DNA transferred from primary to secondary substrate; error bars display ± 1 standard deviation of transfer percentages.

3.2.3. Difference between diluted and undiluted blood

All substrates, except acetate and calico, showed insignificant differences between the diluted and undiluted 5 μL blood samples. Acetate and calico, however, showed a higher quantity ($p = 0.03$) of extracted DNA from diluted than from undiluted blood, indicating that the dilution in TE buffer may have resulted in a small gain of extraction efficiency for these two substrates.

3.3. Concentration efficiency

A loss of DNA during the concentration process was seen in all cases (Fig. 5). Yields of 62%, 39% and 36% were obtained for the initial DNA amounts of 9.2, 55.2 and 552 ng respectively, i.e. DNA concentrations of 0.023, 0.138 and 1.38 ng/μL respectively (Fig. 5).

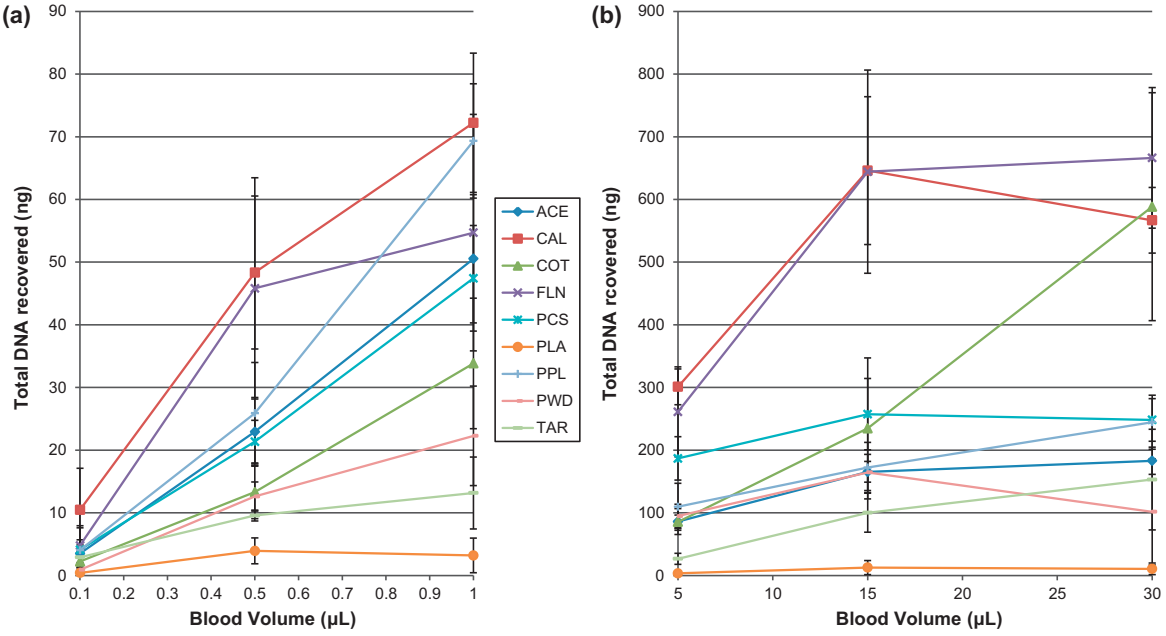

Fig. 4. Extraction efficiency experiment results extracting from (a) 0.1–1 μL of blood and (b) 5–30 μL of blood. Each figure shows the quantity of DNA (ng) obtained from each substrate at the volumes represented on the *x*-axis. (a) The series which contained diluted blood and (b) the undiluted series. Note the *y*-axis scale difference from (a) to (b) to allow greater discrimination among smaller volumes. ACE – acetate, CAL – calico, COT – cotton drill, FLN – flannelette, PCS – polyester/cotton, PLA – plastic transparency, PPL – polyester, PWD – plywood, TAR – tarpaulin.

Table 2
Statistically significant differences between yields for each blood volume on each substrate.

Substrate	Acetate (overall p<0.001)						Calico (overall p=0.002)						Cotton (overall p=0.019)						Polyester (overall p<0.001)						Tarpaulin (overall p<0.001)					
Blood (μL)	0.1	0.5	1	5	15	30	0.1	0.5	1	5	15	30	0.1	0.5	1	5	15	30	0.1	0.5	1	5	15	30	0.1	0.5	1	5	15	30
0.1		ns	ns	ns	*	*		ns	ns	ns	*	*		ns	ns	ns	ns	ns		ns	ns	ns	ns	ns		*	*	*	*	*
0.5	ns		ns	*	*	*	ns		ns	ns	ns	*	ns		ns	ns	ns	ns	*		ns	ns	*	*			ns	*	*	*
1	ns	ns		*	*	*	ns	ns		ns	ns	ns	ns	ns		*	*	ns	*	ns		*	*	*				*	ns	ns
5	ns	ns	ns		ns	ns	ns	ns	ns		ns	*	ns	*	ns		ns	ns	*	ns	ns		ns	ns					ns	ns
15	ns	*	ns	ns		ns	*	*	*	*		ns	ns	*	ns	ns		ns	ns	*	*	*		ns						ns
30	ns	*	ns	ns	ns		*	*	*	*	ns		ns	*	ns	ns	ns		ns	*	*	*	ns							
Blood (μL)	0.1	0.5	1	5	15	30	0.1	0.5	1	5	15	30	0.1	0.5	1	5	15	30	0.1	0.5	1	5	15	30	0.1	0.5	1	5	15	30
Substrate	Flannelette (overall p=0.003)						Poly/cotton (overall p<0.001)						Plastic (overall p=0.002)						Plywood (overall p<0.001)											

Overall *p*-value relates to one-way ANOVA among yields for all volumes for one substrate.
*Significant, ns = not significant ($\alpha = 0.05$).

3.4. Bloodstain characteristics

When conducting the experiments some interesting observations were made on the interactions between blood and the substrates, which may have some implications for interpretation of the results. On fabrics comprising artificial fibres blood tended to spread further and follow a linear pattern (Fig. 6a and f) compared to a more undefined and smaller spread on fabrics made of natural fibres (Fig. 6b–d). An intermediate pattern of spread was seen with polyester/cotton fabric (i.e. fabric containing both types of fibre) (Fig. 6e), which displayed a large, more radial pattern with some linear fringing at its extremities. Blood also dried differently on all three non-fabric substrates, even though the spread was over the same area. Blood on plastic (Fig. 6g) exhibited a 'shattered glass' appearance after drying and tended to fragment on contact and separate from the substrate. While a similar appearance was noted with dried blood on plywood (Fig. 6h), it was tightly attached to the wood and did not separate from it or shatter on contact. Blood dried on tarpaulin formed large flakes (Fig. 6i) which, over 24 h, separated from the substrate and curled.

4. Discussion

4.1. DNA transfer

Our experiments confirm the findings of Goray et al. [8,9] that (i) the quantity of DNA decreases if samples have dried prior to transfer, (ii) that friction increases the amount transferred, and (iii) in general, a highly porous surface acquires more DNA than a non-porous surface, but transfers less as a primary substrate. However, fabrics, other than cotton drill, transferred significantly less DNA than non-porous substrates, and that less DNA was transferred to non-porous than porous substrates, even when thinner fabrics with fibre compositions different to that of cotton were involved.

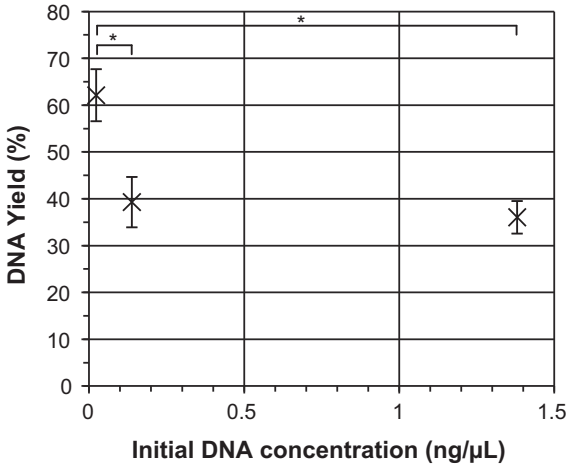

Fig. 5. Concentration efficiency results. Percentage yields are plotted against initial DNA concentrations. Error bars represent percentage yields calculated using ±1 standard deviation of the DNA concentrations after the Amicon step was performed, *a significant difference between the two values indicated ($p < 0.05$).

'Porosity' with respect to substrates has no clear definition. We suggest that porosity relates not to just the macrostructure (i.e. weave in fabrics), but the microstructure as well (the individual fibres). Natural fibres are more porous than artificial fibres, such as polyester and acetate, due to their chemical composition [20]. We hypothesise that DNA transfer is affected by a combination of varying porosities both, macro and micro, which form part of the overall property of the substrate.

Comparing transfer from fabrics consisting of man-made fibres with those made from natural fibres revealed that transfer was overall greater from synthetic fibres. This was not unexpected given their different chemical compositions. No major trend was identified among fabrics as secondary substrates in regards to fibre composition. Comparison between calico and polyester/cotton, two fabrics with the same weave type but different fibre composition, revealed no difference in transfer. Reasons for this similarity may be related to either the thickness of the weaves, as the polyester/cotton fabric comprised a higher number of fibres per centimetre than in calico, or to the specific mix of polyester and cotton fibres. In this study a blend of 65% polyester and 35% cotton was used, which is a ratio common to many fabrics. A different blend of polyester/cotton may produce different results. Further experiments investigating the effect of varying weave thickness and blending of mixed fibres would be valuable.

Microscopic examination showed that blood on polyester fabric pools above the fibre matrix and forms droplets on the surface, rather than diffusing into the fibres as is the case with cotton [21]. This difference in blood absorption by surfaces could help explain why blood is more readily transferrable from polyester and acetate fabrics than from cotton, and may not only have an impact on transfer, but also the quality of extraction and, therefore, assessment of transfer.

Transfer of a dried blood deposit is generally minimal for most substrates, but that from tarpaulin was much higher. This difference is probably related to the large flakes of blood which raise and present themselves on top of the tarpaulin, and are, therefore, readily available for transfer. The formation of these flakes may be related to the hydrophobic properties of the material, polyethylene, from which tarpaulin is made. This hydrophobicity is further illustrated by the finding that approximately half of the DNA on plastic transferred to plywood yet only around 30% transferred to tarpaulin, although this could also be due to the slightly higher porosity of the plywood, and therefore greater absorbency. More research is needed to ascertain how the generation of blood flakes is influenced by the underlying substrate, and also if these flakes are the sole contributing factor to the increase in transfer seen when dried blood is transferred from tarpaulin.

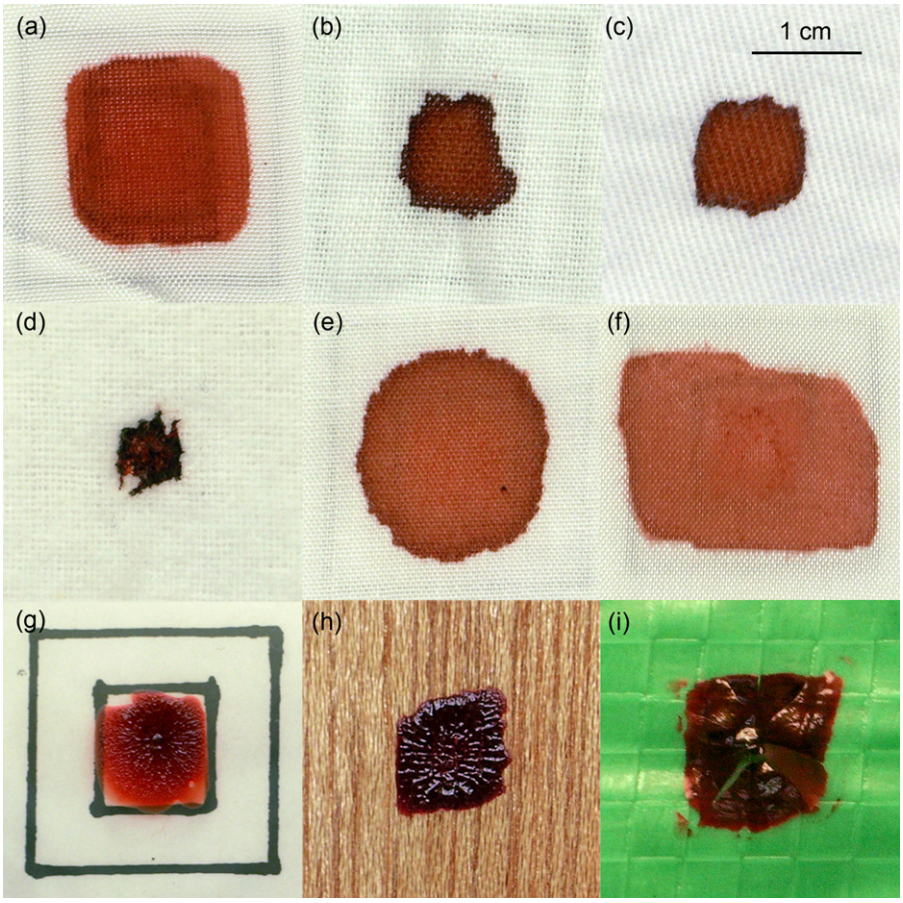

Fig. 6. Morphology of dried blood on all substrates used in experimentation. (a) Acetate, (b) calico, (c) cotton drill, (d) flannelette, (e) polyester/cotton, (f) polyester, (g) plastic, (h) plywood, and (i) tarpaulin.

In casework, horizontal or vertical substrate orientation and/or presence of erosive environmental factors also have an impact on the persistence of a biological sample on a substrate, thus possibly affecting the likelihood of transfer. Time since deposit has been shown to influence the persistence of DNA [22], and it is also important to consider degradation of samples in certain environments. Flaking of blood could also have a direct impact on the persistence of DNA on exhibits. If flakes drop off a substrate prior to sampling, less DNA will be recovered than was deposited on it. The extent to which flaking occurs in casework scenarios is unknown, and should be further investigated for all biological fluids of forensic importance. Recently, Goray et al. [23] found that there can be a significant loss and/or redistribution of DNA containing material from exhibits during packaging and handling. This loss of DNA also needs to be considered when examining amounts of DNA in relation to transfer events.

4.2. Efficiency of DNA extraction

Considerable variation was seen in extraction efficiency of substrates when comparing the amounts of DNA extracted from each. This variation highlights that, even when extracted in the same manner, an amount of DNA obtained from one substrate will not always be comparable to an amount of DNA from another. This observation supports our initial hypothesis that DNA extraction efficiency varies among substrates. It is therefore proposed that correction factors are applied to accommodate this variation in extraction efficiency.

Plastic provided the least DNA after extraction (Fig. 4), indicating that a currently unknown factor affects retrieval of DNA during Chelex extraction. Whether this difference in retrieval among substrates is related to an interaction of blood with substrates or an interaction of the substrate with the extraction method has not been determined; however, as plastic underlay was present in all extraction of fabric samples, the presence of plastic itself does not appear to have a significant chemical effect during extraction. If the lowered quantity of DNA extracted from plastic was related to the fact that flakes of blood are freely available to the solution rather than bound in a matrix, such as extraction from a fabric sample, then a similar reduction in DNA would be expected to be extracted from the similarly non-porous substrate, tarpaulin. Tarpaulin did provide less DNA than most fabrics; nevertheless, the amount of DNA extracted from plastic was always significantly lower than the amount extracted from tarpaulin.

The choice of method has been shown to affect the amount of DNA extractable from a substrate [24,25]. All samples in this experiment were extracted using a modified Chelex protocol. This commonly used method was chosen as it has no inherent maximum threshold of extractable DNA, a feature of DNA binding systems such as DNA IQ (Promega, USA) and ChargeSwitch (Applied Biosystems, USA). Verdon et al. [24], however, have shown DNA IQ to be a more consistent method for extracting DNA from plastic than Chelex. Further research is required to ascertain the reasons for this difference in efficiency between methods.

Extraction efficiency was examined based on the same sampling processes used in transfer experiments to provide a relative assessment of differences between the efficiency of DNA extraction from each substrate. Plywood, however, was sampled

with swabs rather than using direct extraction. Swabbing introduced another process through which DNA could be left behind on the substrate and, as such, the findings may not be comparable to the other substrates in terms of extraction efficiency [26]. The DNA quantity obtained from plywood in our extraction efficiency experiments is still a valid assessment of overall processing efficiency, but relates to a combination of both sampling and extraction efficiency. Therefore, sampling efficiency is yet another variable which should be considered when investigating transfer. Given that DNA sampling methods vary depending on the substrate type encountered and also among laboratories, experiments to examine efficiency of sampling should be undertaken specific to the method(s) being applied.

If assessment of transfer based on DNA quantities is applied to casework, generally there will be no precise information regarding the actual DNA content of biological material from the specific source of the deposit. This lack of knowledge highlights that some degree of uncertainty will inevitably be involved when making assessments of transfer, and this particular contribution to uncertainty will be difficult to accommodate. However, the characterisation of uncertainty relating to calculations of DNA transfer will be assisted by performing rigorous research into both DNA transfer and efficiency of extraction from a variety of biological materials on a range of substrates.

4.3. Efficiency of DNA concentration

A significant loss of DNA was seen during concentration, justifying the need to apply a correction to the value obtained, especially when comparing DNA quantities from samples after concentration with DNA quantities from un-concentrated samples. The efficiency of Amicon concentration devices was greater at lower than higher initial concentrations of DNA, but further testing is required to identify the reason(s) for this. This difference between DNA concentrations may not be universal for each device, and determination of concentration efficiency should be the subject of laboratory in-house studies as no doubt there will be variation among equipment.

When attempting to determine the original amount of DNA present in a sample we suggest correction factors, derived from values obtained during in-house studies of extraction efficiency, be applied to quantities of DNA which have been through a concentration process. In this study only samples containing negligible quantities of DNA were concentrated, and, therefore, to increase the observed amount of DNA after concentration by a factor of 1.6 (based on the yield result of 62% for samples with a low initial DNA concentration) would not have a significant bearing on the final DNA transfer percentages. Nonetheless, in casework and research, particularly when extracting from a relatively large area of substrate and restricted by certain sample handling requirements, such as automated systems, concentration may be necessary for samples with a much higher quantity of DNA. Forensic laboratories investigating issues relating to DNA transfer can only benefit by evaluating the efficiency of their concentration methods.

4.4. Application of correction factors

The standard method of calculating unadjusted transfer percentages (T) was outlined in Section 2.2. To apply correction factors to the transfer data, Eq. (1) is modified:

$$T_A = \frac{D_2}{((E_2/E_1)D_1) + D_2} \times 100 \quad (2)$$

where E_1 is a quantity of DNA extracted from a section of primary substrate in the same manner as, and from a known volume of biological material representative of that which was used in, the transfer samples and E_2 is analogous to E_1, but using a sample of secondary substrate. By including a correction factor (E_2/E_1) derived from a comparison of amounts of extractable DNA from the two substrates used in transfer, this equation reduces bias based on differing extraction efficiencies.

To illustrate the application of the above method, a hypothetical transfer scenario involves a value of 25 ng of DNA extracted from the primary substrate (D_1), and 20 ng of DNA extracted from the secondary substrate (D_2). T is calculated as 44% using Eq. (1), although the actual (hypothetical) amounts of DNA on these substrates after transfer are 50 ng and 100 ng respectively, giving a real-life transfer percentage of 67%. Extraction efficiency experiments performed at the same time provide a ratio of 0.4 for E_2:E_1, allowing adjustment of T via Eq. (2). T_A is calculated to be 67% and concurs with the 'true' value of the transfer percentage.

The correction factors derived using the data obtained from extraction efficiency experiments described in Section 2.4 (Fig. 4), when applied to the quantities of DNA obtained during transfer experiments, led to some highly discrepant results. The 'corrected values' did not concur with our observations and were, therefore, suspected of being more inaccurate than the raw transfer data themselves. One explanation may be that the extraction efficiency experiments performed were not undertaken concurrently with, or with the same blood sample as the transfer experiments. These two issues are potential factors influencing error, and may account for the differences highlighted by the fact that the total DNA extracted from the same substrates but different transfer experiments varied widely as the blood samples differed (data not shown).

To better apply these correction factors in future studies, the use of positive controls for each set of experiments using the same biological fluid used for transfer deposited on the substrates used in the experiments may be useful. Control deposits should be extracted and quantified alongside the transfer experiments to minimise the effects of time as a variable. The quantities obtained would be equivalent to the quantity of DNA that can be extracted from deposits if no transfer step had occurred, and thus, theoretically, relate directly to each applicable extraction efficiency.

Inaccuracy could, however, still be introduced with this form of correction as our data also show that extraction efficiency may vary among different amounts of initial biological material. This latter variable is difficult to accommodate when deriving correction factors, as extrapolating the initial volume of biological material from DNA content requires knowledge of both the initial DNA content of the fluid deposited and the efficiency of extracting that material from its substrate. We propose that when introducing positive controls into experiments, a range of quantities of biological material less than, and equal to, the amount used in transfer also be included in the same control procedures to provide some data on volume dependent extraction efficiency differences (should they exist). These data can subsequently be used to calculate more robust correction factors, or simply to gain insight into how the extraction technique is influenced by the amount of biological material.

An alternative approach to generate correction factors for biological fluid volume data involves the derivation of a standard curve described by the relationship of the quantity of DNA extracted from a substrate and the volume of biological material deposited onto it. The standard curve is subsequently used to extrapolate the volume of fluid initially deposited on a substrate from the quantity of DNA extracted from it. Volumes determined in this manner from both primary and secondary substrates in transfer scenarios are then used to calculate the transfer

percentage:

$$T_A = \frac{E_2}{E_1 + E_2} \times 100 \quad (3)$$

where E_1 is the volume of biological fluid extrapolated from the DNA quantity obtained from the primary substrate, and E_2 is the volume of biological fluid extrapolated from the DNA quantity obtained from the secondary substrate. This method may, however, be limited if the relationship between volume of biological fluid and amount of extracted DNA is complex.

To assist in the long-term investigation of extraction efficiency, we suggest that consideration be given to choosing a substrate to act as a universal reference against which different substrates being examined for casework and research could be compared. Adopting this approach would require analysis of efficiency of extraction from the reference substrate alongside the substrate(s) which has an unknown efficiency of extraction. This approach would generate a comparative database of substrate dependent extraction efficiencies, incorporating different sample types and conditions, potentially across laboratories. At some stage such a database may then be utilised instead of performing case specific experiments.

The effect of the application of correction factors on transfer percentages may vary, depending on how similar the observed DNA quantities from two substrates are, and/or the size of the correction factor. When the DNA quantity extracted from one substrate is greater than an order of magnitude different from the quantity extracted from another substrate, we theorise that applying a correction factor will not alter the transfer percentage as much as if the two quantities of DNA were similar. This difference will be present due to the small overall adjustment in DNA quantity from one substrate relative to the other substrate, unless the correction factor itself is large. This theory, however, needs to be tested empirically.

4.5. Conclusions

Our transfer experiments have shown that different substrates can have a major effect on DNA transfer due to their differing properties, and we suggest that elements of the chemical composition of fibres, the weaves and the thickness of materials are likely to have a role in these differences. More research needs to be conducted into the interaction of forensically relevant biological materials with various substrates, leading to identification and a greater understanding of the microscopic and macroscopic mechanisms which underpin the transfer process. Future research that is focussed on increasing knowledge of the relationship between variables influencing transfer and the amounts of DNA transferred is required. It would also be beneficial to examine further variables likely to influence transfer, including the persistence of DNA, different biological materials (including semen), any environmental effects such as relative humidity, and the effect of the amount of initial deposit. Data generated from experiments examining these variables will improve the assessment of the scenarios proposed during criminal investigations and court proceedings.

Finally, we propose that correction factors, derived using data from the recommended positive control experiments, should be applied in future experiments to reduce some of the error associated with the loss of DNA during processing, particularly where processing of DNA from one substrate and/or biological material has either a higher or lower efficiency than from another. Correction factors should attempt, where possible, to consider differences among sampling and/or extraction efficiency from various initial volumes of biological material, the substrates on which those biological materials were deposited, the types of biological materials involved, and the efficiency of any concentration techniques or DNA clean-up techniques applied downstream.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2012.09.004.

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