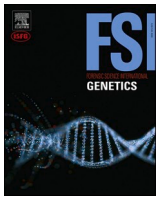
Forensic Science International: Genetics 67 (2023) 102927

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsigen



Comparison of three DNA extraction methods tested on illicit drug-related powders

Amy Griffin [a,*], K. Paul Kirkbride [a], Julianne Henry [a,b], Ben Painter [a,b], Adrian Linacre [a]

[a] College of Science & Engineering, Flinders University, Adelaide 5042, Australia
[b] Forensic Science SA, GPO Box 2790, Adelaide 5000, Australia

ARTICLE INFO

Keywords:
Illicit drugs
Drug profiling
3,4-methylenedioxymethamphetamine
Methamphetamine
Trace DNA
DNA extraction

ABSTRACT

The detection of human DNA on and within illicit drug preparations is novel and a focus of current research. Previous studies have indicated that certain drug-related powders present in illicit drug preparations can interfere with downstream DNA analysis when directly added to the PCR. Therefore, it is important to determine if these drug-related powders are effectively removed during the DNA extraction or whether traces of powder remain to interfere with DNA processing. Three extraction methods were selected to assess their efficiency at removing drug-related powders for downstream processes using DNA from both saliva and touch depositions. This is the first study to compare efficiencies of DNA extraction methods from drug-related powders. The extraction methods compared were the DNA IQ™ System, the QIAamp® DNA Investigator Kit and the combination of a simple lysis step followed by use of the Microcon® DNA Fast Flow device. Saliva was added to dimethylsulfone (DMS), nitrostyrene and PROSOLV® tablet mixture to determine the effect of powder type (based on solubility). Saliva was also added to 0, 50, 200 and 400 mg of DMS to determine the effect of an increase in DMS quantity. Trace DNA was deposited onto DMS using a worn glove approach. These samples were re-tested six months post-DNA deposition and profiled for further comparisons. Ten replicates were conducted for each condition with five replicates of saliva positive controls per method (n = 255 samples). A subset of samples was chemically analysed to determine if DMS was present in the final DNA eluant. The readily soluble DMS did not interfere with any of the extraction methods at lower amounts, however increasing the DMS to 400 mg reduced the relative DNA yields using the Microcon® and Investigator methods. The tablet mixture reduced the relative DNA yield of all three methods, however the nitrostyrene (which was relatively insoluble) only reduced the relative DNA yield of the DNA IQ™. The Investigator method performed the best with the trace samples, followed by the Microcon® method and then the DNA IQ™. DMS was detected in all extracts chemically analysed from the DNA IQ™ and Microcon®, whereas only one sample tested from the Investigator kit contained DMS in the extract and was in a relatively low amount compared to the other samples. Not one kit outperformed the others in all comparisons, however the Investigator kit was the most efficient overall at optimising the DNA yield whilst also removing the powders more effectively.

1. Introduction

Illicit drugs have been reported to contain trace amounts of human DNA which can be extracted and used to yield a DNA profile [1–5]. This biological information could add valuable intelligence to drug-related investigations and complement findings of chemical drug profiling by adding further discrimination between various drug seizure samples or revealing new linkages. Recent research has reported the collection of DNA from the exterior of capsules and tablets [3–5], whereas other previous research explored the composition of DNA within the drug powders themselves [1,2,5]. The inhibitory effects from the presence of these drugs and related compounds on PCR was also investigated, with various compounds reported to inhibit the profile to some degree [6]. The dilution of the drug/DNA mixture was reported to have mitigated these effects for most samples, however this is only a viable option if there is sufficient DNA within the extract. This highlights the importance

* Corresponding author.
E-mail address: amy.griffin@flinders.edu.au (A. Griffin).

https://doi.org/10.1016/j.fsigen.2023.102927
Received 21 November 2022; Received in revised form 4 August 2023; Accepted 7 August 2023
Available online 10 August 2023

of removing the compounds which interfere with the end-to-end DNA profiling process whilst simultaneously maximising the DNA yield during the extraction step.

Illicit drugs such as methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA, also known as ecstasy) are prepared in clandestine laboratories using one of a range of synthetic methods, therefore compounds within the drug preparations will vary between batches/laboratories, depending on accessibility and availability of compounds at the time of manufacture and the particular synthetic method employed [7–10]. As such, there is no way to know exactly what may be within the powders until chemical analysis is conducted. Nevertheless, the final preparation will generally contain the active illicit drug along with synthesis by-products and residues of precursor, other adulterants (compounds which are pharmacologically active) and diluents (powders which are added to increase the total weight of the material to enhance profit and aid in dosage control) [7–10]. The salt form of the drugs will generally be present, as it is easily soluble in water (thus aiding intravenous consumption), whereas the base form and certain intermediates are less soluble in water and in the case of methamphetamine and MDMA are relatively volatile liquids [11]. Tablets are another, distinct powder type, as they generally contain additional ingredients specific to the function of the tablet, including the incorporation of binder-fillers, lubricants, superdisintegrants and glidants [12,13]. Whilst some preparations of illicit tablets incorporate home-made mixtures where the various components have been purchased separately and subsequently mixed, there have been reports of the purchase of excipients used by the pharmaceutical industry such as all-in-one composites where all relevant components are present, and the active ingredient just needs to be added (Personal Communication, S. Jaswal, Health Canada, 2019).

Due to the uncertainties surrounding the composition of drug preparations which could be encountered in case samples, three different drug-related powders were selected which aimed to represent the different possible powder types (water soluble, water insoluble and tablet mixture). The potential interference in the DNA quantification and amplification observed in the presence of some drug-related powders also meant that the drugs themselves were not a suitable substrate for accurately determining the DNA yields between the methods (as the DNA yields were often found to be suppressed in the quantification in a previous study [6]). As most drugs seized in the salt form in capsules and as loose powders/crystals are water soluble, dimethylsulfone (DMS) was used as a substitute for a drug, and as it is one of the most common cutting agents added to a range of drugs it is an important drug-related powder to investigate in its own right [14]. Nitrostyrene is a precursor which can be synthesised in clandestine laboratories and then used in further reactions to eventually synthesise methamphetamine [15]. After its synthesis this compound could be left drying on trays within clandestine laboratories accumulating potentially informative DNA. As bulk amounts or traces of nitrostyrene are often seized during clandestine laboratory investigations it is therefore a useful drug-related powder to investigate on that basis alone, however it is also useful as an example of the relatively insoluble compounds which may be encountered in other drug samples. PROSOLV® tablet mixture (a commercial composite of four components required for optimal tablet pressing) was used as an example of possible excipients present within illicit tablets which may be more difficult to extract DNA from compared to DMS or nitrostyrene as the solubility may differ for some components more than others within the composite powder. Therefore, the three drug-related powders selected for this study were DMS (water soluble), nitrostyrene (water insoluble) and PROSOLV® tablet mixture.

This study also compares three DNA extraction methods: the DNA IQ™ System, the QIAamp® DNA Investigator kit and the combination of a simple lysis step followed by use of the Microcon® DNA Fast Flow device. The DNA IQ™ System (a magnetic resin-based system) and the QIAamp® DNA Investigator kit (filter-based system utilising the varying solubility of DNA in different solutions) are two commercial kits commonly employed in operational laboratories for routine DNA extraction [16]. The use of the Microcon® DNA Fast Flow device (another centrifugal size exclusionary filter-based system) is used for the concentration of nucleic acids and can be used as a simple DNA purification and concentration method following a cell lysis step [2,17]. These methods were chosen as they are representative of what is used routinely; however, they also use different chemistries to concentrate and purify the DNA. This may be important for extracting DNA from drug powders, as the mechanism which concentrates the DNA could lead to co-elution of certain drug-related powders, where a different kit with a different chemistry may not. It is hypothesised that most compounds present in illicit methamphetamine and MDMA preparations, such as tablets, powders and capsules, will either dissolve and be removed with the DNA extraction buffers, or that they will remain solid and can be pelleted out from the solution, allowing any DNA to be purified and concentrated without the presence of illicit drugs and related compounds for downstream PCR.

The aim of this study was to test this hypothesis by comparing the quantity and quality of the DNA extracted from DMS, nitrostyrene and PROSOLV® tablet mixture using the DNA IQ™ System, the QIAamp® DNA Investigator Kit and the Microcon® DNA Fast Flow device. The effect of the amount of DMS added and the efficiency of purifying the DNA extract (whether the DMS remained in the final eluate) were also investigated to determine if one extraction method outperformed the others with both high and low quantity and quality sources of DNA.

2. Materials and methods

2.1. Ethics approval

Ethics approval was obtained from the Social and Behavioural Research Ethics Committee of Flinders University (reference 4235). DNA samples were obtained and analysed with informed consent.

2.2. Sample preparation

The three powders selected to compare with three extraction methods were: 1) DMS (Nourishme Organics, Victoria, Australia) (a readily water soluble and common diluent), 2) phenyl-2-nitropropene (P2NP) (Merck, Darmstadt, Germany), which will be referred to as nitrostyrene (relatively water insoluble and a precursor to amphetamines) and 3) PROSOLV® EASYtab SP (JRS PHARMA, Rosenberg, Germany), a tablet mixture sold for pharmaceutical use without an active ingredient. All samples were tested in replicates of 10 as outlined in Table 1.

Firstly, 200 mg of DMS and PROSOLV were used and 50 mg nitrostyrene (due to limited availability). Two microlitres of fresh, neat saliva were added to each powder in a 1.5 mL microcentrifuge tube (saliva was vortexed for at least 15 s prior to aliquoting to allow effective homogenisation). The amount of DMS was also varied to test the effect this had on the extraction methods by adding 2 μL of saliva to 0 mg, 50 mg, 200 mg, and 400 mg of powder. Positive controls (2 μL of saliva only) were included in each extraction batch in replicates of five to provide insight into the reproducibility of each method and allow relative comparisons between methods. One negative control was included in every extraction batch for each method where no saliva or powder was added. Negative controls of each powder type with each method were also included where the powder was added without saliva to determine the amount of background DNA present in each powder.

Trace DNA samples were prepared following the “inverted glove method” with 200 mg DMS [5]. Briefly, a volunteer was asked to wear a pair of latex gloves (Bastion, Adelaide, Australia) for approximately four hours. These gloves were then inverted and worn on top of new gloves by the researcher who used the inside of the worn gloves to deposit DNA onto the DMS by rubbing it for approximately 10 min. This was not to replicate how DNA may be added to drug powders but to simply add trace amounts of DNA in dry form to allow homogenisation of DNA

Table 1
Summary of experimental design which was completed for each DNA extraction method (using the DNA IQ™ System, QIAamp® DNA Investigator kit and Microcon® DNA Fast Flow device) using either saliva or trace DNA spiked into dimethylsulfone (DMS), PROSOLV tablet mixture or nitrostyrene. The total number of samples n = 255 does not include negative controls.

Source of DNA	DMS		PROSOLV		Nitrostyrene	
	Weight of sample (mg)	Number of replicates	Weight of sample (mg)	Number of replicates	Weight of sample (mg)	Number of replicates
Saliva (2 μL)	0	5	0	5	0	5
	50	10				
	200	10	200	10	50	10
	400	10				
Touch	200	10				
Touch (six months since deposition)	200	10				Total: 255 samples

within the powder. This powder (200 mg per replicate) was then transferred to appropriate tubes for extraction with the different methods. Because of the sample preparation methodology, equivalent positive controls could not be prepared. The powder was tested and then stored in a container in the laboratory and re-tested 6 months after DNA was deposited to determine its stability over time. Only the 6-month samples were subjected to DNA profiling as they would represent the most realistic type of sample that may be encountered in real drug samples (in terms of DNA quantity and quality compared to saliva sources).

2.3. DNA analysis

The extraction methods used were the DNA IQ™ System (Promega, Wisconsin, USA), QIAamp® DNA Investigator Kit (QIAGEN, Victoria, Australia) and the Microcon® DNA Fast Flow device (Merck Millipore Ltd., Cork, Ireland). A summary of the protocol followed for each method is outlined in Table 2. The extraction of samples using the DNA IQ™ System (referred to as DNA IQ from hereafter) was carried out following the "Small Sample Casework Protocol" for processing solid samples with the "Preprocessing Using Lysis Buffer" protocol, using an initial lysis volume of 850 μL with 20 μL Proteinase K (18 mg/mL). The use of a spin basket was not necessary and therefore this step was omitted, however the tubes were still centrifuged, and the supernatant was transferred to a new tube as per the protocol. Following on from this the "DNA Purification Using DNA IQ™ Resin" protocol was performed with a final elution in 60 μL. The extraction of samples using the QIAamp® DNA Investigator Kit (referred to as Investigator from hereafter) was carried out following the QIAamp® DNA Investigator Handbook protocol for isolating total DNA from surface and buccal swabs, with 400 μL ATL, 20 μL Proteinase K (18 mg/mL) and 400 μL AL lysis buffer and a final elution in 60 μL. The samples extracted using the initial lysis step followed by use of the Microcon® DNA Fast Flow device (referred to as Microcon from hereafter) were processed following the guidelines in the User Guide (PRO4305, Rev. 06/18) after an initial incubation in 850 μL of sodium bicarbonate solution (150 mM, Merck) with 20 μL of Proteinase K (18 mg/mL) for 30 min at 56 °C [2,17]. Following this the supernatant was transferred 500 μL at a time to the Microcon column and centrifuged at 500 x g for 15 min. The filter was then rinsed three times with 200 μL water at 500 x g for 12 min. The elution buffer (20 μL) from the DNA IQ kit was added to the filter which was then inverted into a new tube and centrifuged for 3 min at 1000 x g. With the remaining liquid present and the elution buffer added, the final volume ranged between 60 and 90 μL. With all extraction methods, if any powder remained insoluble after the initial incubation, the tubes were centrifuged at 14,000 rpm for 1 min and the supernatant transferred to a new tube (DNA IQ) or onto the filter (Investigator and Microcon).

All extracts were quantified using the Investigator® Quantiplex Pro RGQ (QIAGEN) with the Rotor-Gene Q following the manufacturer's protocols. The 6-month trace samples were profiled using VeriFiler™ Plus PCR Amplification Kit (ThermoFisher Scientific, Victoria, Australia) also following the manufacturer's protocols. Either 500 pg of DNA was added to the PCR or up to 17.5 μL for samples where the DNA quantity was less than 0.029 ng/μL. The PCR products were separated on a 3500 Genetic Analyzer (ThermoFisher Scientific) with 8.5 μL Hi-Di

Table 2
Summary of protocols used for each extraction method.

		DNA IQ	Microcon	Investigator
Lysis	Buffer type	• Prepared lysis buffer (1 μL of 1 M DTT for every 100 μL of Lysis Buffer) • Proteinase K (18 mg/mL)	• Sodium bicarbonate solution (150 mM) • Proteinase K (18 mg/mL)	• Buffer ATL • Proteinase K (18 mg/mL) • Buffer AL (contains guanidine hydrochloride)
	Lysis volume	850 μL prepared lysis buffer, 20 μL Proteinase K	850 μL sodium bicarbonate, 20 μL Proteinase K	400 μL ATL, 20 μL Proteinase K, 400 μL AL
	Incubation temperature	70 °C	56 °C	56 °C with ATL and Proteinase K, followed by 70 °C after AL is added
	Incubation time	30 min	30 min	1 h with ATL and Proteinase K, followed by 10 min after AL is added
DNA capture	DNA capture method	Paramagnetic particles in form of resin	Ultracel® low binding regenerated cellulose filter	Silica-based membrane (QIAamp MinElute column)
	Volume used for DNA capture	Entire lysate mixed with 7 μL resin	Entire lysate passed through filter (500 μL at a time)	Firstly, lysate is mixed with 200 μL ethanol then entire lysate is transferred 700 μL at a time through column
Purification	Buffer type	Wash Buffer (contains ethanol and isopropanol)	Water	AW1, AW2 (both contain ethanol), ethanol
	Buffer volume	100 μL	200 μL	500 μL AW1, 700 μL AW2, 700 μL ethanol
	Number of washes	1 x prepared lysis buffer 3 x wash buffer	3	1 x AW1 1 x AW2 1 x ethanol
	Final elution volume	60 μL	60–90 μL	60 μL

formamide, 0.5 μL 600 LIZ® size standard and 1 μL PCR products. Profiles were analysed using GeneMapper® ID-X (version 1.4) (Thermofisher Scientific) at an analytical threshold of 50 relative fluorescence units (RFU). STRmix™ (version 2.8.0) was used to deconvolute the profiles and calculate likelihood ratio (LR) values using settings specific to VeriFiler™ (available upon request). The point estimate LR values based on the Australian Caucasian population are reported.

2.4. Chemical analysis

To determine if DMS was still present in the final DNA extracts, a subset of the replicates with the highest and lowest reported DNA quantity within the 50 mg and 400 mg DMS samples for each extraction method were selected to be analysed chemically using gas chromatography-mass spectrometry (GCMS). The extracts (50 μL) were mixed with ethanol (50 μL) and analysed using an Agilent 7890 gas chromatograph equipped with a 5975 triple-axis mass spectral detector and an Agilent 7693 autosampler. Chromatographic separation was conducted using a HP5-MS column (dimensions 29.4 m x 250 μm x 0.25 μm) and the mass spectral data were recorded in electron ionisation mode at 70 eV (scan range *m/z* 40–550). Analysis was performed in split mode (split ratio 1:10) with an injection port temperature of 280 °C and an injection volume of 1 μL. Helium was used as carrier gas at constant flow (1.5 mL/min). The temperature program was as follows: initial temperature at 60 °C, held for 1 min, then ramped at 15 °C/minute to 120 °C and held for a further 3 min.

DMS was first analysed separately to determine the retention time (3.7 min) and then the DNA IQ and Investigator elution buffers were analysed separately to verify that there were no other peaks detected which might co-elute with the DMS. The subset of samples was then analysed, and the peak areas were recorded.

2.5. Data analysis

Mann-Whitney U Tests, Kruskal-Wallis Tests and Wilcoxon Signed Rank Tests (paired) were conducted in RStudio (version 1.3.1093) with a statistical significance reached at $p < 0.05$. A Post-Hoc Dunn's test was carried out following significance indicated by the Kruskal-Wallis Tests to determine specifically which groups were significantly different to one another. Mann-Whitney U tests were used when two groups were compared, such as the DNA yields from each powder type spiked with saliva for each DNA extraction kit compared to their respective controls. The relative DNA yield was then calculated for all saliva samples using the following equation:

$$Relative\ \ DNA\ \ yield\ \ (\%) = \frac{DNA\ \ yield\ \ of\ \ sample(ng)}{Average\ \ DNA\ \ yield\ \ of\ \ respective\ \ positive\ \ controls(ng)} \times 100$$

where the DNA yield of each sample was divided by the average DNA yield of the five positive controls extracted in the same batch as the sample and multiplied by 100 to convert to a percentage. The Kruskal-Wallis test was used when there were three or more categorical, independent groups compared, such as for all saliva samples when comparing the relative DNA yields between the three extraction methods for each powder type and amount of DMS. For the trace DNA samples, the total DNA yield was obtained by multiplying the DNA yield (ng/μL) by 60 μL, which was used as a conservative amount for the Microcon samples where the final volume varied.

The Kruskal-Wallis Test was used to compare the DNA yield of the trace samples (0 and 6 months) between the three methods and then a Wilcoxon Signed Rank Test (paired) was used to compare the DNA yields of the 0- and 6-month samples within each method (as the samples are of the same origin and retested 6 months later and are therefore paired, or dependant). Both the number of donor alleles and subsequent LR of the 6-month samples were compared between the three extraction methods using a Kruskal-Wallis Test.

3. Results and discussion

3.1. Physical behaviour of each drug-related powder during DNA extraction

The DMS dissolved into the lysis buffer for all three methods during incubation, however after returning to room temperature the DMS in the Investigator samples crystallised out (for the 200 mg and 400 mg samples), forming a pellet after centrifugation. As expected, the PROSOLV samples did not completely dissolve for any of the methods, however after visual assessment, it was evident that some of the components in the tablet mixture had dissolved as the pellet was less than the initial amount of powder added. The nitrostyrene melted in all samples during the incubation, however it formed a bi-phasic system and crystallised out after returning to room temperature. Some of the nitrostyrene must have dissolved into the DNA IQ lysis buffer however, as following incubation and centrifugation, the lysis buffer mixture had changed from a colourless, clear liquid to a clear light yellow (the nitrostyrene is bright yellow) and the nitrostyrene pellet was smaller than for the other methods. The washes carried out in the DNA IQ method allowed the final eluate to return to the expected colourless clear liquid.

3.2. DNA recovery from drug-related powders spiked with saliva

The DNA yield for the control samples ranged from an average of 280 pg to 44 ng depending on the saliva sample used with that batch. Although, as expected, there was intervariation observed with the DNA yield from the control samples between batches (where this variation was attributed to the saliva source), there was minimal intravariation of DNA yield from the control samples within the same batch. For this reason, only the relative DNA yield will be discussed when comparing samples where saliva was the DNA source. As these DNA extractions were conducted manually, due to the sheer number of samples tested with each extraction method, it was not possible to test all drug-related powder types in one batch. To avoid any bias with the varying DNA concentration of saliva sources, the powder types and other variables were often split between batches (for example five replicates in one batch and the remaining five replicates of the same powder type in another). This would have minimised the problem but the variation in initial DNA concentration of the saliva samples remains a limitation of this DNA source and is part of the reason trace DNA from the hands was used later in this research. No DNA was detected in any of the negative control samples. The Internal Control (IC) within the quantification PCR was not flagged for any samples within this study, indicating inhibition did not occur for any samples. Possible degradation was indicated within the quantification data for some samples, as outlined in Table S1, however this generally correlated with a low DNA yield or where the control samples also had possible degradation indicated.

The relative DNA yields from the powders spiked with saliva varied between powder type and extraction method when compared to the positive controls with no powder added (Fig. 1, Table S1). The average relative DNA yields where 200 mg of DMS was added were 107%, 83%

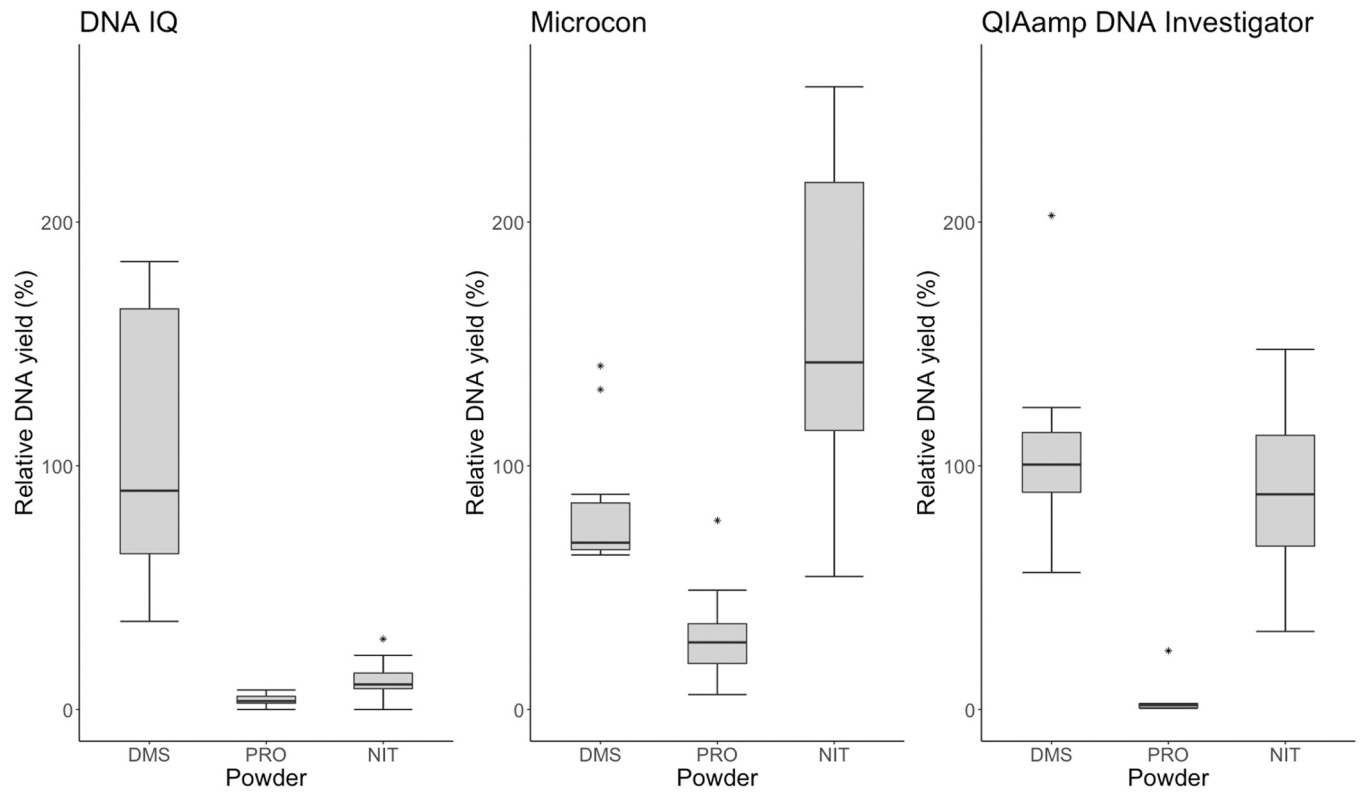


Fig. 1. Boxplots (n = 10 replicates) of the relative DNA yields (%) when saliva was added to 200 mg dimethylsulfone (DMS), 200 mg PROSOLV (PRO) and 50 mg nitrostyrene (NIT). Results are shown for DNA IQ (left), Microcon (middle) and QIAamp® DNA Investigator (right). The relative DNA yield was determined by comparing each yield to the average of the five positive controls run in the same extraction batch. The horizontal line within the boxplots represents the median, and any outliers are represented by a black asterisk.

and 105% for DNA IQ, Microcon and Investigator, respectively, and were not deemed significantly different compared to their respective positive controls for any of the extraction methods tested (Mann-Whitney U test; p-value 0.7949, 0.0536 and 0.5687 for DNA IQ, Microcon and Investigator, respectively). There was, however, a greater distribution in the relative DNA yield between replicates from the DMS using the DNA IQ samples compared to the other methods (Fig. 1, Table S1). The DNA yields from the PROSOLV powder were less than the positive controls using all three methods. The Microcon method produced the highest average relative DNA yield of 31%, compared to 4% for the DNA IQ and Investigator methods; these differences were all deemed significant (Mann-Whitney U test; p-value 0.0002 for all methods). DNA yields from the nitrostyrene samples were deemed significantly different to the positive controls when using the DNA IQ and Microcon methods (Mann-Whitney U test; p-value 0.0002 and 0.0375, respectively). DNA yields were lower than the positive controls when using DNA IQ with an average relative DNA yield of 12%, but were higher using Microcon, with an average relative DNA yield of 158%. The DNA yields were not statistically different when using Investigator with an average relative DNA yield of 87% (Mann-Whitney U test; p-value 0.7949). As mentioned above, there was likely some nitrostyrene still present in the lysis buffer following incubation with the DNA IQ. This could have interfered with the binding of DNA to the resin causing it to be lost during the first wash step. A likely explanation for the high variability and higher relative DNA yield (over 100%) for the Microcon samples with nitrostyrene is that the nature of this method requires the supernatant to be transferred to the top of the microcon, which is then centrifuged to allow the liquid to flow through whilst concentrating the DNA on the filter. The final elution volume is difficult to reproduce with an accurate volume as it depends on how well the liquid flows through the Microcon. If some of the nitrostyrene remained in the supernatant and was transferred to the Microcon (as was sometimes observed), or if a small amount dissolved during the incubation and then recrystallised during centrifugation, it may have interfered with how quickly the liquid could flow through and require more centrifugation compared to the control, possibly further concentrating the DNA. It is also possible that traces of the nitrostyrene (which is bright yellow in appearance) were present in the final eluate, which interfered with the fluorescence detection during the quantification step and inflated the reported DNA concentration. A similar phenomenon has been observed due to the presence of humic substances [18].

When comparing the relative DNA yields there was no significant difference between the three methods for the 200 mg DMS samples (Kruskal-Wallis test; p-value 0.6374). The relative DNA yields from the PROSOLV samples were significantly different between the three DNA extraction methods (Kruskal-Wallis test; p-value 0.0001). The PROSOLV samples extracted with the Microcon method produced higher relative DNA yields compared to both the DNA IQ and Investigator kits; this difference was deemed significant using the Post-Hoc Dunn's test (p-value 0.0015, p-value 4.7×10^{-5}, respectively) but not significantly different between the DNA IQ or Investigator methods (p-value 0.187). Statistical differences were observed when comparing the nitrostyrene samples between extraction methods (Kruskal-Wallis test; p-value 1.53×10^{-5}). The DNA IQ performed worse than the Investigator and Microcon methods when comparing the nitrostyrene samples which was deemed significant with the Post-Hoc Dunn's test (p-value 0.003 and 3.3×10^{-6}, respectively), however no significant difference was observed with the nitrostyrene samples between the Investigator and Microcon methods (p-value 0.094).

DMS was selected for further studies based on the following factors: in the initial experiment where 200 mg was added, it did not significantly affect the relative DNA yield for any of the extraction methods, it is commonly used with various illicit drugs as a diluent, and it is also most likely to persist with the DNA to the final eluate due to its high

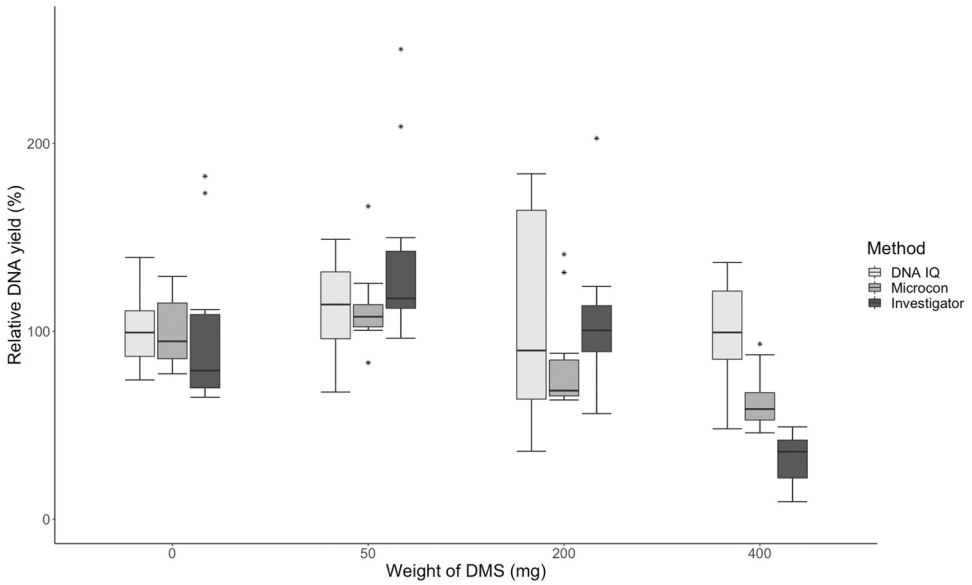


Fig. 2. Boxplots (n = 10 replicates) of the relative DNA yields (%) when saliva was added to varying amounts of dimethylsulfone (DMS) using the DNA IQ, Microcon and QIAamp® DNA Investigator extraction methods. The relative DNA yield was determined by comparing each yield to the average of the five positive controls run in the same extraction batch. The horizontal line within the boxplots represents the median, and any outliers are represented by a black asterisk.

water-solubility compared to the insoluble nitrostyrene and tablet mixture which can be pelleted out. When 0 mg, 50 mg, 200 mg and 400 mg of DMS was combined with saliva and extracted using the DNA IQ method, the average relative DNA yields were 100%, 111%, 107% and 99%, respectively, which were not deemed significantly different to one another (Kruskal-Wallis test; p-value 0.8293, Fig. 2). For the Microcon method, as the amount of DMS increased from 0 mg to 50 mg, 200 mg and 400 mg the relative DNA yield decreased from 100% to 107%, 83% and 63%, respectively; these differences were deemed significant (Kruskal-Wallis test; p-value 0.0003). A Post-Hoc Dunn's test revealed that significant differences were obtained using the Microcon method when 400 mg of DMS was added compared to 0 mg and 50 mg (p-value 8.7×10^{-4} and 6.9×10^{-5}, respectively). Significant differences in the relative DNA yields were observed between the amounts of DMS added using the Investigator kit (Kruskal-Wallis test; p-value 1.1×10^{-5}), where the average relative DNA yield extracted from the 400 mg DMS samples of 32% was less than each of the 0 mg, 50 mg and 200 mg samples, with average DNA yields of 100%, 140% and 105%, respectively. All of these were deemed significantly different to the 400 mg samples (Post-Hoc Dunn's test, p-values 0.002, $8.8 \times 10^{-7}$and 5.0×10^{-4}, respectively).

In real drug preparations, the amount of DNA likely to be present will presumably vary on a case-by-case basis, and therefore it would be best to add as much of the preparation as possible to maximise the DNA yield without diminishing returns. Based on these results, adding 400 mg does begin to cause a decrease in DNA yield for the Microcon and Investigator methods and 200 mg of preparation may be closer to optimal (Fig. 2). A possible explanation for why the DNA IQ was not impacted by the increase of DMS compared to the other two methods is due to how it binds the DNA using the resin and magnet compared to centrifugation. From preliminary studies, it was observed that some substrates (including powder which had not dissolved) could interfere with the resin mixing with the lysis buffer/ DNA mixture and subsequently moving to the magnet (data not presented in this study). Due to the readily soluble nature of DMS within the lysis mixture, this was not an issue in these experiments. It could also explain why the PROSOLV and nitrostyrene samples extracted with the DNA IQ had lower relative yields. If traces of the powders were not effectively removed during the initial centrifugation and some remained in the supernatant (particularly with the observed yellow solution from the nitrostyrene samples) which was then mixed with the resin, it could have caused inefficient binding of the DNA with the resin, or with the resin to the magnet. Incompatibilities with the DNA IQ chemistry have been observed in previous literature and were determined to be caused by competition for DNA binding sites using the resin [19,20]. It is unknown however, if there are any instances in other laboratories where the resin was unable to move to the magnet as observed during the manual extractions in this study.

3.3. DNA recovery from drug-related powders spiked with trace DNA

The use of saliva as a source of DNA was to provide a reliable, reproducible, and known amount of good quantity and quality of DNA to the extractions for comparisons between the methods. This is not, however, a realistic representation of the quantity, form, and quality of DNA likely to be present within illicit drug preparations [21]. For this reason, a method using worn gloves was performed to deposit trace amounts of DNA onto powder in a dry form, allowing homogenisation of the DNA within the powder. Immediately after the trace DNA was deposited onto the DMS (0 months), the DNA IQ method had lower DNA yields (average of 46 pg) than the Microcon and Investigator methods (averages of 110 pg and 192 pg, respectively), which was deemed a significant difference (Kruskal-Wallis test; p-value 0.0002, Fig. 3). The Post-Hoc Dunn's test identified a significant difference between the DNA IQ and Investigator methods (p-value 3.4×10^{-5}), but not between the DNA IQ and Microcon, or Microcon and Investigator methods (p-value 0.050 and 0.029, respectively). Six months post-deposition, the extractions were repeated and the DNA IQ and Microcon both reported lower DNA yields than the Investigator method (averages of 47 pg, 41 pg and 104 pg, respectively), which was deemed significant (Kruskal-Wallis test; p-value 0.0003). The Post-Hoc Dunn's test revealed that the differences between both the DNA IQ and Microcon methods were significantly different to the Investigator method, but not between one another (p-value 8.9×10^{-4}, 2.5×10^{-4} and 0.731, respectively). The DNA IQ yields did not significantly change after the DNA was on the DMS for 6 months (Wilcoxon Signed Rank test (paired); p-value 0.1527), however the Microcon and Investigator yields were both significantly different (Wilcoxon Signed Rank test (paired); p-value 0.0051 for both methods) when directly comparing the 0- and 6-month samples within each method (where the yields were lower after 6 months).

The difference between the extraction methods is further highlighted

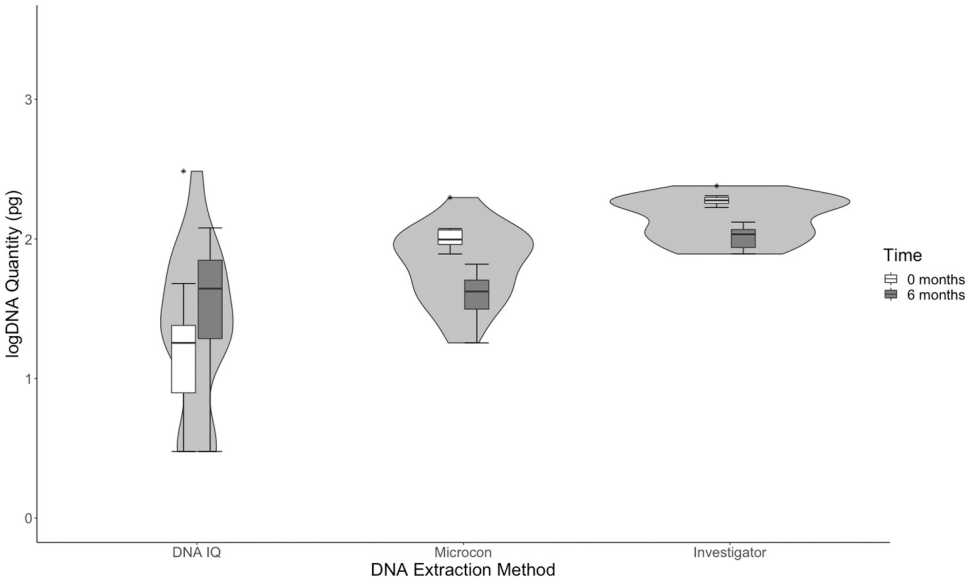


Fig. 3. Boxplots of the log DNA quantity (pg) when trace DNA added to 200 mg of dimethylsulfone (DMS) was extracted immediately (0 months) and 6 months after deposition with the DNA IQ, Microcon and QIAamp® DNA Investigator extraction methods (n = 10 replicates). Violin plots are overlayed combining the two time points, where the width of the violin represents the density of samples falling within that log DNA quantity. Samples for which DNA was not detected are displayed at log 0.5. The horizontal line within the boxplots represents the median, and any outliers are represented by a black asterisk.

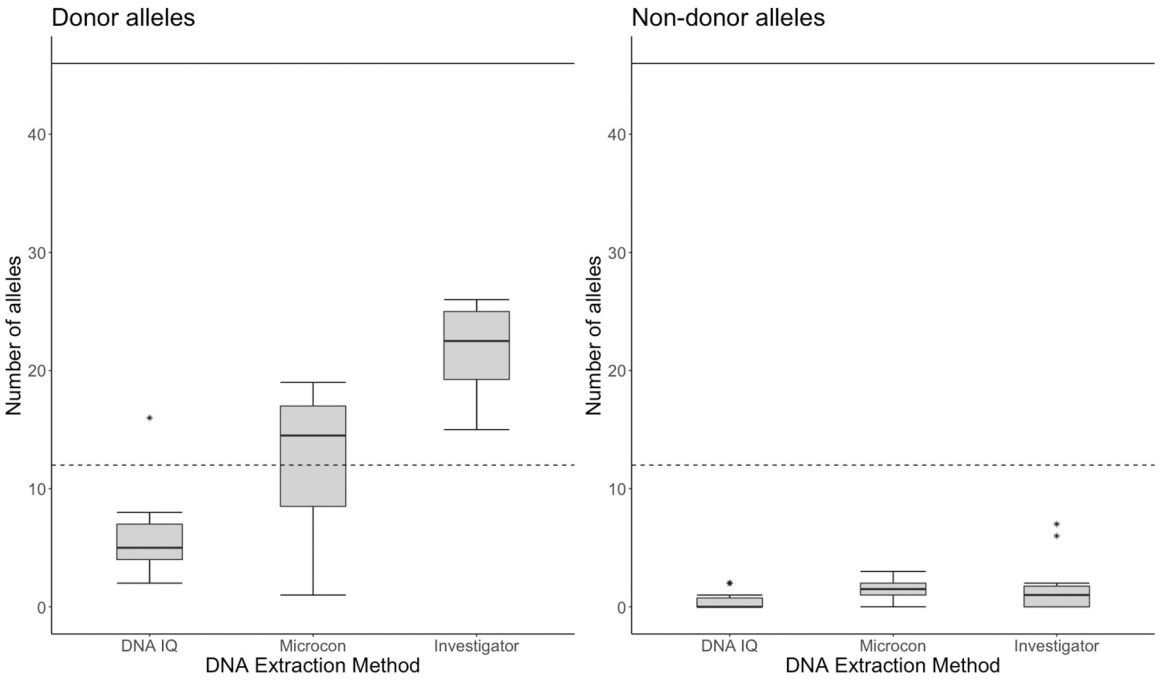


Fig. 4. Boxplots (n = 10 replicates) of the number of donor and non-donor alleles detected in the trace DNA samples of dimethylsulfone (DMS, 200 mg) extracted 6 months post-deposition using the DNA IQ, Microcon and QIAamp® DNA Investigator methods. The dashed line at 12 alleles represents the threshold for a profile to be considered informative in South Australia. The solid black line at 46 alleles represents a full profile. The horizontal line within the boxplots represents the median, and any outliers are represented by a black asterisk.

in the subsequent profiling of the 6-month trace samples (Figs. 4 and 5). The number of donor alleles detected using the Investigator method was higher than that of the DNA IQ and Microcon; this difference was deemed significant (Kruskal-Wallis test; p-value 8.7×10^{-5}), with an average of 22, 6 and 13 donor alleles detected, respectively. Further analysis with the Post-Hoc Dunn's test confirmed the number of donor alleles from both the DNA IQ and Microcon methods were significantly different to the Investigator method, but not between one another (p-value 1.7×10^{-5}, 0.011 and 0.077, respectively). The Investigator and Microcon methods yielded enough alleles (at least 12) to be uploaded to the National Criminal Investigation DNA Database (NCIDD) in South Australia, however the DNA IQ did not.

Although the trace DNA was deposited onto the DMS by handling the powder with the inside of gloves which had been worn for approximately four hours after handwashing, surprisingly there was some non-donor DNA detected that could not be attributed to contamination. Negative controls of just the powder without additional DNA were tested and did not yield any alleles. It is not unusual for trace DNA to contain non-donor alleles [22], however, it is interesting that even after four hours of wearing gloves, full profiles of the donor were not yielded but non-donor alleles were detected. It is possible that these non-donor alleles could be due to stochastic effects due to the low amounts of template DNA present within the sample. The number of non-donor alleles was not significantly different between the methods (Kruskal-Wallis test;

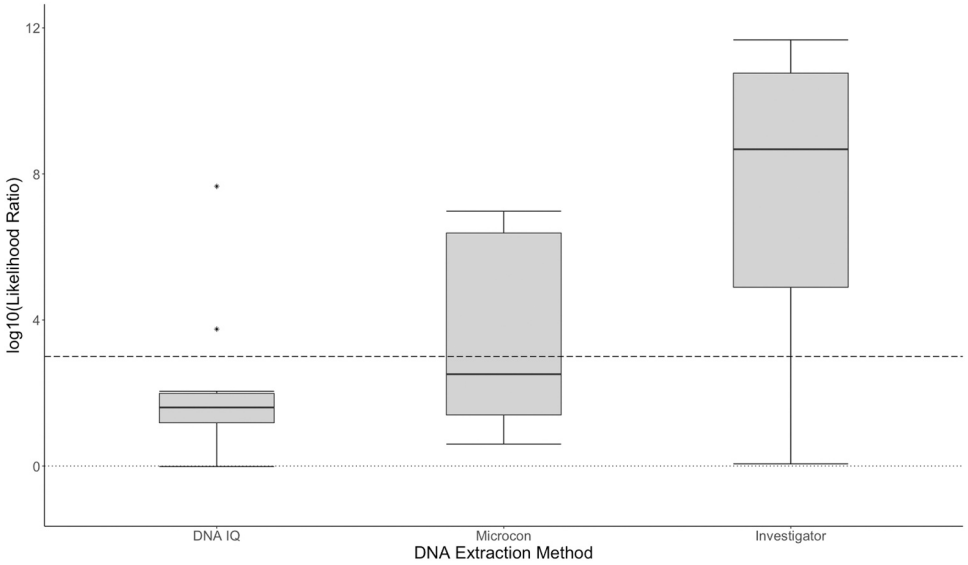


Fig. 5. Boxplots (n = 10 replicates) of the log10 likelihood ratio values generated from the trace DNA samples of dimethylsulfone (DMS, 200 mg) extracted 6 months post-deposition using the DNA IQ, Microcon and QIAamp® DNA Investigator methods. The dashed line at log10 LR= 3 represents the threshold where anything above LR= 1000 indicates strong support for the inclusion of the individual as a profile contributor [23]. The dotted line at log10 LR= 0 represents the threshold where anything above LR= 1 indicates support for the inclusion of the individual as a profile contributor, and anything less indicates support against it. The horizontal line within the boxplots represent the median, and any outliers are represented by a black asterisk.

p-value 0.1106) and the average did not exceed 2 alleles for any method.

The LR values generated from the trace samples were statistically different between the methods (Kruskal-Wallis test; p-value 0.01129, Fig. 5). The LR values of the Investigator samples were higher than the Microcon samples and both the Investigator and Microcon samples were higher than the DNA IQ samples. Whilst the difference between the Investigator and DNA IQ methods was deemed significant (Post-Hoc Dunn's test; p-value 0.003), the difference between the Investigator and Microcon or the Microcon and DNA IQ was not (Post-Hoc Dunn's test; p-value 0.042 and 0.374, respectively). The average DNA IQ LR values sit within the verbal equivalent of "moderately strong support" (100−1000), the Microcon method with "strong support" (1000−10,000) and the Investigator method of "extremely strong support" (>1000,000) for the inclusion of the donor as a profile contributor [23]. Based on these results, with trace amounts of DNA present, the difference in the efficiency of each method drastically affects the outcome and informativeness of the subsequent profile.

3.4. GCMS results

After comparison of the DNA yields between the three methods with the three powder types, different amounts of DMS and with both saliva and trace DNA, it was still unknown whether some methods were more efficient at removing the powder and purifying the DNA than others. Theoretically, the compounds which remained insoluble following the lysis step (such as the PROSOLV and nitrostyrene for all methods and DMS for Investigator 200 mg and 400 mg samples) will be removed with centrifugation for all methods. However, some or all the compounds which may be soluble (such as DMS) in the buffers could have persisted by binding to the resin for the DNA IQ or the spin filters for the Investigator and Microcon methods. The persistence of some drugs or other compounds into the final eluate could interfere with the downstream DNA analysis [6]. A previous study which added compounds and DNA directly to the PCR (without an extraction) determined that some drugs and related compounds do interfere with the efficiency of the PCR,

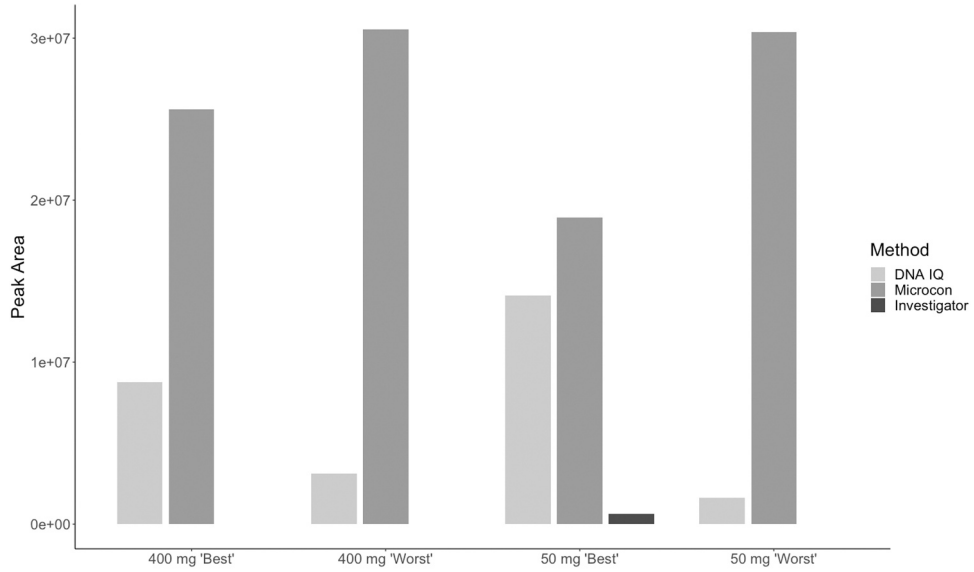


Fig. 6. GCMS peak areas of dimethylsulfone (DMS) detected in extracts using the DNA IQ, Microcon and QIAamp® DNA Investigator methods. Of the ten samples for each method, the replicates with the highest ('best') and lowest ('worst') reported DNA yields from 400 mg and 50 mg DMS which had 2 μL neat saliva added were analysed.

however this interference was reduced following dilution of the solution prior to amplification (effective if enough DNA is present in the sample) [6]. It is therefore important to determine if these compounds are likely to be effectively removed during the extraction. DMS was tested in this previous study and did not interfere with the PCR as much as most other compounds analysed, therefore it was chosen for this experiment as a common diluent likely to be present with many drugs (whilst still behaving most similarly to the drugs compared to PROSOLV or nitrostyrene), but also to allow direct comparison between the extraction methods whilst also providing the opportunity for accurate downstream DNA results. A subset of the DMS saliva samples were selected based on the DNA quantity following extraction with the three methods. The replicates with the highest and lowest DNA quantities for the 50 mg and 400 mg DMS samples were selected, equating to four samples per method to be chemically tested for the presence or absence of DMS in the final eluates (Fig. 6).

DMS was detected in all DNA IQ and Microcon extracts using GCMS (with a peak area between 1.6×10^6 and 3×10^7 Units) but in only one Investigator extract (in a relatively low amount of 6.5×10^5 Units). The peak area of DMS in the Microcon extracts was higher than that for the DNA IQ extracts (Fig. 6). Interestingly, the amount of DMS persisting in the DNA IQ and Microcon extracts did not appear to depend on how much DMS was added to begin with but were instead proportional and inversely proportional to the DNA yields, respectively. That is, the replicate with the highest DNA yield with DNA IQ also was found to contain the highest amount of DMS, whereas the highest DNA yield with the Microcon was found to contain the lowest amount of DMS (Fig. 6). This could indicate that, with the DNA IQ, the DMS may interact with the DNA or resin, whilst still allowing the DNA to bind to the resin. Therefore, when less DNA was obtained, there was also less DMS present in the extract. In contrast, it is possible that the presence of DMS interfered with the performance of the Microcon, but again not enough to be significantly different from the controls with the saliva samples. As the DMS remained soluble in the buffers for the DNA IQ and Microcon methods, this could explain why there was more present in the final DNA extract as the entirety of the DMS was put through the extraction and was more likely to persist. In contrast, with the Investigator method, the DMS crystalised out of solution in the 400 mg DMS samples but not with 50 mg. Again, this was not a significant issue for the DMS samples with the Investigator method, however if other drugs which are also soluble in the lysis buffer behave in a similar manner, then this could be a problem for downstream DNA analysis.

4. Conclusion

This study has indicated that each of the three DNA extraction methods tested has different benefits and drawbacks depending on the drug-related powder type, amount of powder added to the extraction and amount of DNA present. For example, there was no difference in the relative DNA yields between the methods for the extraction of saliva when 200 mg of DMS was added, however with 400 mg both the Investigator and Microcon methods were negatively impacted. The PROSOLV tablet mixture which did not completely dissolve following the lysis step in any method caused decreased relative DNA yields compared to the controls for all three methods. The extraction of DNA from saliva with nitrostyrene caused a significantly lower relative DNA yield for DNA IQ, a higher DNA yield from the Microcon method, and it did not affect the DNA yield for the Investigator kit. Overall, for trace DNA (as expected in real casework seizures), the Investigator kit performed the best in terms of DNA yield and subsequent LR generated for the donor. The difference in the number of alleles detected and subsequent likelihood ratio could ultimately be the difference in successfully identifying a person of interest.

The results also indicated that the Investigator method was the most effective in removing the DMS from the final DNA extract. DMS is likely to be present in many illicit drug seizure samples as it is a common cutting agent. Although previous research has indicated that it does not interfere with downstream DNA analysis, it is preferable to remove all non-DNA impurities where possible. It is likely that many other drugs and drug-related powders may co-elute in a similar way to DMS and potentially interfere with downstream DNA analysis. Further studies are needed to investigate the effect of a larger range of compounds on these extraction methods, especially compounds that are ionizable at the pH values used for lysis and extraction.

Whilst this investigation demonstrated that the DNA IQ, Microcon and Investigator methods could all be used to extract DNA from illicit drug preparations, the Investigator kit is recommended as it recovered the highest amount of DNA from all drug-related powders tested, even at lower quantities and quality, whilst also removing the drug-related powder most effectively.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by an Australian Government Research Training Program Scholarship and funding was provided by the Attorney General's Department through Forensic Science SA and the Ross Vining Research Fund.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2023.102927.

References

[1] L.A. Burgoyne, D.E. Catcheside, K.P. Kirkbride, The bioprofiling of illicit drugs, National Drug Law Enforcement Research Fund (NDLERF), Hobart, 2008.
[2] L.A. Burgoyne, D.E. Catcheside, K.P. Kirkbride, C. Pearman, Assessment of the utility of obtaining human profiles from drug seizures, National Drug Law Enforcement Research Fund (NDLERF), Hobart, 2012.
[3] A. Griffin, K.P. Kirkbride, J. Henry, B. Painter, A. Linacre, DNA on drugs! A preliminary investigation of DNA deposition during the handling of illicit drug capsules, Forensic Sci. Int.: Genet. 54 (2021), 102559.
[4] A. Griffin, K.P. Kirkbride, J. Henry, B. Painter, A. Linacre, DNA on drugs (part 2): an extended study into the transfer and persistence of DNA onto illicit drug capsules using realistic scenarios, Forensic Sci. Int.: Genet. 60 (2022), 102740.
[5] A. Griffin, K.P. Kirkbride, J. Henry, B. Painter, A. Linacre, Recovery of integrated and surface trace DNA from illicit drug tablets, Forensic Sci. Int.: Genet. 61 (2022), 102772.
[6] A. Griffin, J. Henry, P. Kirkbride, B. Painter, A. Linacre, A survey of the effects of common illicit drugs on forensic DNA analysis, Forensic Sci. Int. (2022), 111314.
[7] J. Broséus, S. Baechler, N. Gentile, P. Esseiva, Chemical profiling: a tool to decipher the structure and organisation of illicit drug markets: an 8-year study in Western Switzerland, Forensic Sci. Int. 266 (2016) 18–28.
[8] M. Collins, Illicit drug profiling: a historical perspective, Drug Test. Anal. (2021).
[9] S. Meola, P. Esseiva, What is the future of illicit drug profiling in Switzerland? Condemned to disappear or forgotten treasure, Drug Test. Anal. (2021).
[10] A. Popovic, M. Morelato, C. Roux, A. Beavis, Review of the most common chemometric techniques in illicit drug profiling, Forensic Sci. Int. 302 (2019), 109911.
[11] UNODC, World Drug Report, 2022, United Nations publication, 2022.
[12] S.E. Bell, D.T. Burns, A.C. Dennis, L.J. Matchett, J.S. Speers, Composition profiling of seized ecstasy tablets by Raman spectroscopy, Analyst 125 (10) (2000) 1811–1815.
[13] C. Weyermann, R. Marquis, C. Delaporte, P. Esseiva, E. Lock, L. Aalberg, J. S. Bozenko Jr, S. Dieckmann, L. Dujourdy, F. Zrcek, Drug intelligence based on MDMA tablets data: I. Organic impurities profiling, Forensic Sci. Int. 177 (1) (2008) 11–16.
[14] S.G. Toske, T.D. McKibben, Monitoring methamphetamine in the United States: a two-decade review as seen by the DEA methamphetamine profiling program, Drug Test. Anal. (2021).
[15] S.G. Toske, J.L. Brown, E.E. Miller, M.Z. Phillips, S.C. Kerr, P.A. Hays, Recent methamphetamine profiling trends: tracking the nitrostyrene method used for P2P production, Forensic Chem. 13 (2019), 100140.

[16] K.W.Y. Chong, Z. Thong, C.K.C. Syn, Recent trends and developments in forensic DNA extraction, Wiley Interdisciplinary Revie, ws: Forensic Sci. 3 (2) (2021), e1395.
[17] Merck, User guide: microcon centrifugal filter devices, PRO4305, Rev. 06/18 (2018).
[18] M. Sidstedt, L. Jansson, E. Nilsson, L. Noppa, M. Forsman, P. Rådström, J. Hedman, Humic substances cause fluorescence inhibition in real-time polymerase chain reaction, Anal. Biochem. 487 (2015) 30–37.
[19] C.J. Frégeau, A. De Moors, Competition for DNA binding sites using Promega DNA IQ™ paramagnetic beads, Forensic Sci. Int.: Genet. 6 (5) (2012) 511–522.
[20] N. Laurin, F. Célestin, M. Clark, D. Wilkinson, B. Yamashita, C. Frégeau, New incompatibilities uncovered using the Promega DNA IQ™ chemistry, Forensic Sci. Int. 257 (2015) 134–141.
[21] M.H. Toothman, K.M. Kester, J. Champagne, T.D. Cruz, W.S. Street IV, B.L. Brown, Characterization of human DNA in environmental samples, Forensic Sci. Int. 178 (1) (2008) 7–15.
[22] M. Goray, S. Fowler, B. Szkuta, R. Van Oorschot, Shedder status—an analysis of self and non-self DNA in multiple handprints deposited by the same individuals over time, Forensic Sci. Int.: Genet. 23 (2016) 190–196.
[23] ENFSI, ENFSI guideline for evaluative reporting in forensic science, 2015.