

Investigating the detection limits of scent-detection dogs to residual blood odour on clothing



LaTara Rust^{a,*}, Katie D. Nizio^a, Matt P. Wand^b, Shari L. Forbes^a

^aUniversity of Technology Sydney, Centre for Forensic Science, P.O. Box 123, Broadway, NSW 2007, Australia

^bUniversity of Technology Sydney, School of Mathematical and Physical Sciences, P.O. Box 123, Broadway, NSW 2007, Australia

ARTICLE INFO

Article history:

Received 5 March 2018

Received in revised form 28 May 2018

Accepted 30 May 2018

Available online 1 June 2018

Keywords:

Blood evidence

Crime scene clean-up

Scent-detection dogs

Luminol

Solid phase microextraction (SPME)

Comprehensive two-dimensional gas chromatography (GC×GC)

ABSTRACT

Blood-detection dogs are trained to locate blood evidence and search for potential crime scenes in cases where a cadaver may not be present. The locations of crime scenes are often ambiguous and evidence may not always be obvious during initial processing. In cases of foul play, a criminal may attempt to clean biological evidence from a crime scene; however, trace evidence that appears invisible to the naked eye may still be detectable. For example, it has been reported anecdotally that blood-detection dogs are capable of detecting blood on clothing that has been washed up to five times, or on surfaces which have been scrubbed clean. This study aimed to investigate the baseline detection limits of blood-detection dogs and cadaver-detection dogs to latent blood evidence on washed clothing and to compare the dogs' responses to current presumptive chemical and analytical techniques. Blood was deposited onto cotton swatches and washed up to five times with a standard household washing machine. Following washing, the cotton swatches were allowed to dry and presented to blood-detection and cadaver-detection dogs during law enforcement training. Replicates of these samples were tested with luminol spray and analysed using headspace solid phase microextraction – comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry (HS-SPME-GC×GC-TOFMS). Results indicated that the olfactory system of blood-detection and cadaver-detection dogs is a viable complementary technique to presumptive chemical tests and more sensitive than current scientific instrumentation, with some of the dogs able to detect blood after five washes but HS-SPME-GC×GC-TOFMS only able to detect blood after two washes or less. This limit of detection could likely be lowered for the dogs with further and more consistent training. Luminol was similarly able to detect blood washed up to five times, which indicates that the scenting abilities of these dogs can provide investigators with valuable information that may be overlooked during preliminary searches in cases when chemical testing is unsuitable. This study highlights the importance of training blood-detection and cadaver-detection dogs for increased sensitivity to blood so that evidence collected at a scene can be further analysed for greater evidentiary value.

© 2018 Elsevier B.V. All rights reserved.

Abbreviations: ¹D, First dimension; 1D, One-dimensional; ²D, Second dimension; 3D, Three-dimensional; EDTA, Ethylenediaminetetraacetic acid; F_{crit} , Critical F value; HCA, Hierarchical cluster analysis; HS-SPME-GC×GC-TOFMS, Headspace solid phase microextraction – comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry; LCV, Leuco-crystal violet; NIST, National Institute of Standards and Technology; PC-1, First principal component; PC-2, Second principal component; PC-3, Third principal component; PCA, Principal component analysis; PDMS/DVB, Polydimethylsiloxane/divinylbenzene; ppt, Parts-per-trillion; S/N , Signal-to-noise ratio; TIC, Total ion current; VOC, Volatile organic compound.

* Corresponding author.

E-mail addresses: Latara.Rust@gmail.com (L. Rust), katiednizio@gmail.com (K.D. Nizio), Matt.Wand@uts.edu.au (M.P. Wand), Shari.Forbes@uts.edu.au (S.L. Forbes).

1. Introduction

Locating blood at a crime scene is an integral step in recreating the events of a crime, identifying victims or suspects, establishing secondary crime scenes, identifying links between individuals, locations and/or objects and ascertaining potential weapons. Law enforcement can employ various methods when processing a crime scene for blood evidence including chemical presumptive tests and crime scene dogs (i.e. cadaver-detection and blood-detection dogs). These methods are used to identify areas of interest for further processing with confirmatory tests and collection of samples.

The locations of crime scenes are often ambiguous and evidence may not be visible to investigators during initial processing of a

scene. In cases of foul play, a criminal may attempt to wash away biological material such as blood at a crime scene so as to avoid leaving trace evidence [1–3]. However, trace evidence that appears invisible to the naked eye may still be detectable through sensitive chemical presumptive tests [2]. It has been reported that DNA can be extracted from bloodstains cleaned with chlorinated bleach, soap or disinfectants, and thus having effective tools to locate latent blood can be valuable for an investigation [3].

There are several presumptive chemical tests for blood that can be utilised at a crime scene, each with their own suitable applications. Typically these chemical tests fall under two methods of interaction with blood: 1) an interaction of peroxidase activity from the haem in haemoglobin which results in the emittance of light when observed in darkness (such as luminol or fluorescein); or 2) oxidation in the presence of haemoglobin which results in a colour change reaction (such as phenolphthalein (also known as the Kastle–Meyer test), tetramethylbenzidine, leucomalachite green and orthotolidine) [4]. Phenolphthalein is the most commonly used colour change test due to its high sensitivity and suitability to latent stains; however, it is prone to false positive results to common items such as rust, metal salts and various vegetables [4,5]. Other colour change tests have higher specificity to blood, however, are unsuitable for diluted bloodstains and testing large surfaces for latent traces of blood. For this reason, luminol (a chemiluminescence test) and other commercially similar products are utilised more commonly by investigators [4,6,7].

Luminol is a common presumptive test which has been employed in the field of forensic science for over 40 years, and is considered an important tool for locating bloodstains at crime scenes [7]. Compared to other available chemical presumptive tests it is considered the most suitable for blood due to its high sensitivity, specificity and non-interference with subsequent DNA analysis [8]. However, luminol requires a dark room to visualise stains, can have false-positive reactions to bleach and other cleaning products, and may further dilute already weak stains for extracting DNA [4]. Quantitative studies have tested the efficiency of luminol to detect bloodstains on surfaces which have been cleaned several times with water and bleach on non-porous tiles [9], porous brick and cotton fabric [2]. One study demonstrated that haemoglobin is still detectable with luminol after 10 wipes with water and 2 wipes with bleach on tiles [9], while another found that haemoglobin was still detectable on cotton and brick that had been washed with bleach until invisible to the naked eye [2].

Other studies have shown that bloodstains on various types of fabric, both synthetic and natural, are less likely to be retained after washing with chlorine-containing detergents when tested with phenolphthalein and orthotolidine presumptive tests, but that fabrics containing cotton have a strong affinity to blood [10]. A study by Adair and Shaw in 2005 [1] investigated the sensitivity of luminol and leuco-crystal violet (LCV) used to develop latent bloodstains from clothing washed up to five times with common cleaning products. Latent bloodstains were detected on all washed shirts after application of both luminol and LCV, with luminol producing superior results overall [1].

It is important to consider that the use of luminol and other blood presumptive reagents may not always be suitable to every crime scene, particularly expanses of area outdoors or indoors where spraying large surfaces could be time and cost inefficient [7]. This is equally important in scenarios when a perpetrator may attempt to conceal a crime using harsh cleaning agents that could interfere with the effectiveness of these chemical tests. Implementing cadaver-detection dogs and blood-detection dogs as a screening technique in clean-up scenarios to detect bloodstain evidence can allow investigators to process a crime scene more efficiently and maintain any evidentiary value of bloodstains.

Blood-detection dogs are a subset of cadaver-detection dogs in Australia that are trained solely on human blood. Cadaver-detection dogs, which can be trained on various human tissues including human blood, are common worldwide and are deployed in cases of mass disaster, missing persons or homicide where locating human remains is their primary objective. Blood-detection dogs are trained only to locate blood and are more commonly deployed at crime scenes to identify areas of interest, potential murder weapons or track the movement of suspects or victims to secondary scenes. In many countries including Australia, both blood-detection and cadaver-detection dogs can be utilised by law enforcement, and thus this study observed the deployment suitability of both types to washed blood in cases of a concealed crime.

If latent blood that has been cleaned can be detected with presumptive chemical tests, then an odour associated with blood may also be detectable. It is not known if cadaver-detection dogs or blood-detection dogs are able to detect blood evidence which has been washed from clothing, with no reported data in the literature. Some law enforcement agencies have reported, anecdotally, that their dogs could detect blood on clothing that had been washed repeatedly in biological detergent [11]; however, these reports need to be tested and validated.

In recent years there has been an increased interest in elucidating the scenting capacity, mechanisms and sensitivity limits of various scent-detection dogs used to search for drugs, explosives and human remains [12–19]. It is unclear how these dogs are able to target specific scents (i.e. whether it is individual compounds or a mixture of compounds that elicit a response) and their true limits of detection. Of these studies, there is limited research that has directly compared chemical testing of biological tissue with the scenting capability of blood-detection and cadaver-detection dogs in a forensic context, with most studies restricted by the number of dogs available to obtain suitable replicates [17,18].

A study by Skalleberg & Bouzga [17] compared crime scene dogs and presumptive tests in relation to the detection of trace semen and bloodstains in outdoor environments on coniferous forest ground. The authors determined that crime scene dogs were able to detect blood volumes as small as 0.1 mL up to 32 h post-deposition, with the presumptive tetrabase test detecting volumes as low as 0.05 mL up to 48 h post-deposition, demonstrating the dogs' potential as screening tools which have similar sensitivity to presumptive chemical tests [17]. The ability to extract DNA at these volumes was most significant if collected within the first 24 h which emphasises the importance of implementing efficient screening methods such as blood-detection dogs to streamline the investigation [17].

The Netherlands law enforcement in 2013 also compared the sensitivity of two cadaver-detection dogs to three common blood presumptive tests (luminol, tetrabase and Kastle–Meyer) [18]. Fresh blood was diluted up to 4000 times and deposited in 0.2 mL volumes onto smooth (vinyl) and rough (carpet) surfaces. The presumptive tests outperformed the dogs when exposed to the smooth surface, but the dogs were able to perform much better with the rough surfaces in comparison to the chemical tests [18]. The authors highlighted that the type of surface is an important consideration when implementing various detection methods, and that in some cases indications by scent-detection dogs may not be able to be confirmed with current technology [18]. This is an important consideration for investigators as dogs are often reported to be more sensitive than current analytical instrumentation [20,21].

This study aims to investigate and establish the baseline limit of detection of blood- and cadaver-detection dogs when compared with the presumptive chemical test luminol by tracking the

persistence of blood on cotton swatches washed up to five times to replicate a scenario where a suspect attempts to clean clothing containing blood. Volatile organic compound (VOC) profiling was performed using HS-SPME-GC×GC-TOFMS based on previous research conducted by the authors for blood VOCs [22]. Luminol was chosen for presumptive chemical testing due to its reported high sensitivity and specificity to blood, and because it is a common tool in most crime scene kits [8]. The results of this study will assist law enforcement with training recommendations for cadaver-detection and blood-detection dogs for the discovery of latent blood evidence, and provide scientific evidence to support the deployment of these dogs for crime scene searches.

2. Materials and methods

2.1. Experimental design

Whole human blood was collected from a single individual to prevent inter-subject variability. The blood was collected by qualified phlebotomists via venipuncture into BD Vacutainer[®] Tubes with Lavender BD Hemogard[™] Closures (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), containing an EDTA (ethylenediaminetetraacetic acid) additive to prevent clotting during transfer of sub-samples. The donor was encouraged to maintain their typical diet and hygiene practices prior to blood collection in order to ensure an accurate reflection of natural blood composition for analysis. The donor, a healthy female, aged 25, reported that she was not taking any medications at the time of collection. This study was approved by the human ethics committee at the University of Technology Sydney (UTS HREC #2013000132).

After collection, blood was transferred in 1 mL volumes onto 5 cm × 5 cm cotton swatches cut from a white 100% cotton t-shirt (Kmart, Sydney, NSW, Australia). This volume and surface were used to replicate the scenario of blood deposition on clothing transferred during the process of a violent crime, and was found to be the most suitable for the detection of blood volatiles from porous surfaces as based on a previously optimised method [22]. After the blood was deposited onto the surface of the cotton swatches, the bloodstains were allowed to dry over a 12-hour period before washing. Samples prepared for odour profiling were sealed prior to analysis in individual 250 mL aluminium tins (Morris McMahon & Co Pty Ltd, Sydney, NSW, Australia).

After removing replicates to represent unwashed positive controls (unwashed blood) the remaining bloodstained cotton swatch replicates were washed alone (i.e. in isolation from any other bulk clothing) in a standard top-load washing machine on cold wash (Hoover 550 M, Sydney, NSW, Australia) with a non-phosphate and low-fragrance Earth Choice Ultra Concentrate laundry powder (Woolworths, Sydney, NSW, Australia) for up to five wash cycles. The plant-based laundry powder was chosen for the study to limit the number of odorants interfering with sample analysis in order to provide a baseline understanding of the sensitivity of the methods utilised in this study, which is hypothesised to be quite low in comparison to biological detectors (i.e. scent-detection dogs). The brand also represented a common household detergent that would be found in most homes and easily accessible to perpetrators. No bleach agents were investigated in this study, and thus it is recommended that future work further explore this variable in respect to crime-scene clean-up scenarios of blood for scent-detection dogs.

After each wash cycle, the swatches were air-dried and five replicates from each wash cycle were sealed in individual aluminium tins for subsequent analysis (Table 1). The remaining swatches were placed back into the washing machine for the next cycle and the process was repeated until all five washes were completed. Additional cotton swatches containing no bloodstains (i.e.

washed negative controls) were also washed with this method in order to account for the VOC profile of laundry powder used in this study (Table 1). These control swatches were washed separately to the bloodstained swatches on the same day, with an empty wash cycle completed between each wash in order to ensure no cross-contamination occurred. Cotton swatches which had not been washed (i.e. unwashed negative controls) were also included as a control to account for background VOCs from the cotton t-shirt (Table 1).

2.2. Blood-detection and cadaver-detection dog trials

Four cadaver-detection dogs and one blood-detection dog were provided by local law enforcement for testing in this study (Table 2) and were approved by the animal ethics committee at the University of Technology Sydney (UTS ACEC #2014000213). Blood-detection dogs have only recently been introduced into Australia. Dog trials took place over three training sessions in a six-month period, with each training session occurring approximately every two months. Due to operational commitments, not all dogs were available for all three training sessions but were present for a minimum of two. Detailed information of each dog and their attendance can be seen in Table 2.

The dog trials took place at the local law enforcement training facility and followed their standard procedures in order to provide baseline sensitivity results. The week preceding each training session, a new set of blood and control samples were prepared and washed. The training set-up involved three rows of cement blocks containing tins (marked 1–48) which were placed in a U-shaped formation around the training room (refer to Fig. 1), with 16 tins in each row. These tins contained either blood samples (target odours) or negative controls (unwashed and washed) and were sealed by a lid containing perforated holes to allow for permeation of odours (Fig. 1). Between each testing session, these teams continued to train with their regular blood training aids (cadaver, fresh and aged blood on various surfaces) as per their operational requirements.

Within each search there was a total of eight target odour tins: three unwashed positive controls (i.e. fresh blood on cotton) and one cotton swatch replicate containing blood washed from 1 to 5 times. The remaining tins were negative control tins containing three lots of washed negative controls washed from 1 to 5 times (for a total of 15 washed negative controls), and 25 replicates of plain cotton swatches (i.e. unwashed negative controls). The position of target odours and control tins in the U-shaped formation was chosen using a random number generator with the following restrictions: each row contained one unwashed positive control (i.e. unwashed blood) and each row also contained one replicate each of the 1–5 washed negative controls. Target odours and negative control tins were handled by separate individuals wearing gloves to reduce contamination of odours, and all tins and lids were wiped with fresh paper towels to create a common scent across target odour and negative control tins. The order that the dogs searched at each training session was also randomised.

These experiments were single-blind studies where the handler leading the dog was blind to the location of the target odours; however, the positions of the target odours were provided to a separate officer responsible for rewarding the dog. This reward officer was positioned at a distance from the handler and dog, away from direct view, with the reward concealed until a correct alert was performed and the handler called the alert. All scent-detection dogs used in this study were rewarded with play and performed on-leash searches. Each dog had their own unique alert ranging from a sit-and-stare to feet tapping, with all classified as passive alerts.

Table 1

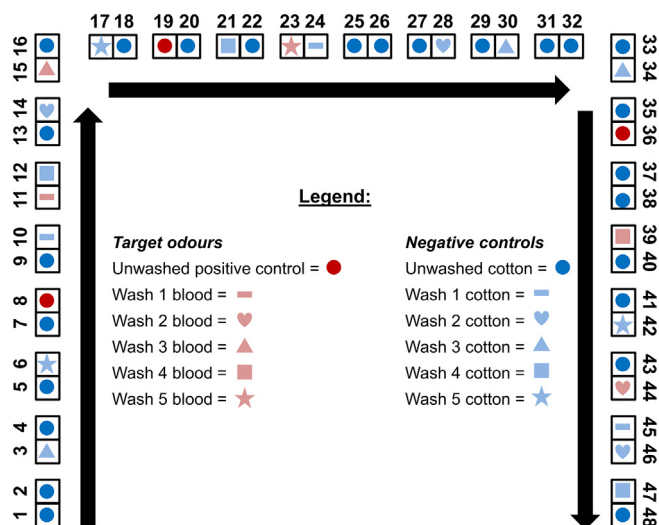
Summary of experimental and control swatches prepared and examined for each analysis tested in this study.

	Number of Swatch Replicates			
	Unwashed Positive Controls	Washed Samples	Unwashed Negative Controls	Washed Negative Controls
Dog Trials	×3	×3 (per wash cycle)	×25	×3 (per wash cycle)
Luminol	×5	×5 (per wash cycle)	×1 (per wash cycle)	×1 (per wash cycle)
Odour Profiling (HS-SPME-GC×GC-TOFMS)	×5	×5 (per wash cycle)	×1 (per wash cycle)	×1 (per wash cycle)

Table 2

Detailed list of the cadaver-detection and blood-detection dogs utilised in this study with recorded attendance at each training session.

Team	Age	Breed	Gender	Cadaver- or blood-detection dog	Years of experience	Training Session		
						1	2	3
A	6	English Springer Spaniel	Female	Cadaver-detection	5	✓		✓
B	6	English Springer Spaniel	Female	Cadaver-detection	5	✓	✓	✓
C	7	German Shepherd	Male	Cadaver-detection	5		✓	✓
D	4	Labrador	Female	Blood-detection	2.5	✓		✓
E	4	English Springer Spaniel	Male	Cadaver-detection	1	✓	✓	

**Fig. 1.** An example schematic of one scent line-up of unwashed and washed blood samples, and unwashed and washed negative controls randomised for this study.

For each search, the handler led the dog along each tin once and their responses were recorded as either a correct alert (true positive response to the target odour) or an incorrect alert (false negative response to the target odour) or false positive response in the absence of the target odour). Final alerts and behaviour changes, such as partial alerts (pausing over tins, head-flicks or attempting to go back to previous tins), from each dog from each dog were identified by the handler and any false positive alerts were also recorded for future training improvements. A false positive response is classified as an alert without the target odour being present, and a false negative response is classified as the lack of an alert despite the presence of the target odour.

At each training session the dogs were tasked with searching the U-shaped formation three times (once with each dog before the dogs repeated their second and third search), with tins rearranged between every search and between every dog. This resulted in a total exposure to three replicates of cotton containing blood washed 1–5 times and nine replicates of unwashed blood, with the potential for 24 correct alerts for each training session. Target odour tins had their lids replaced between every search and between every dog and all tins (target and negative controls) were wiped clean with fresh paper towel as previously described.

Target odour and negative control tins were replaced with new tins between rounds of searches (i.e. after first, second and third searches) and again wiped with fresh paper towel.

2.2.1. Statistical analysis

In order to evaluate the collective potential of these dogs to detect visible and latent blood in this baseline study, a number of odds ratios were calculated to determine the studied effects (such as number of washes, training and detection type) on their probability of detection. Calculating an odds ratio allows the comparison of the relative odds of the dogs to locate the blood on cotton given the interaction of the effects previously stated. An odds ratio estimate of exactly 1 means that the effect did not affect the odds of the dogs locating cotton with blood. An odds ratio estimate greater than 1 means that the effect was associated with higher odds of the dogs locating cotton with blood, and an odds ratio less than 1 means that the effect was associated with lower odds of the dogs locating cotton with blood.

The data collected from the blood-detection and cadaver-detection dog trials was analysed using logistic regression [23]. The main model was as follows:

$$\begin{aligned} \log(\text{odds of detecting target odour}) = & \beta_0 + \beta_1(\text{number of washes}) \\ & + \beta_2(\text{indicator that dog is A}) + \beta_3(\text{indicator that dog is A}) \\ & \times (\text{number of washes}) \\ & + \beta_4(\text{indicator that dog is B}) + \beta_5(\text{indicator that dog is B}) \\ & \times (\text{number of washes}) \\ & + \beta_6(\text{indicator that dog is C}) + \beta_7(\text{indicator that dog is C}) \\ & \times (\text{number of washes}) \\ & + \beta_8(\text{indicator that dog is E}) + \beta_9(\text{indicator that dog is E}) \\ & \times (\text{number of washes}) \\ & + \beta_{10}(\text{indicator of dog having 2 training sessions}) \\ & + \beta_{11}(\text{indicator of dog having 3 training sessions}) \end{aligned} \quad (1)$$

where 'log' denotes natural logarithm and

$$\text{odds of detecting target odour} = \frac{\text{probability of detecting target odour}}{\text{probability of missing target odour}}$$

In model (1) the main effects are:

- the number of washes treated as a quantitative variable,
- the particular scent-detection dog with dog D, the blood-detection dog, as the baseline,

- the number of training sessions treated as a categorical variable with one training session as the baseline.

The model also allows for interaction effects between the number of washes and the scent-detection dog. For example, the odds ratio of a dog detecting the target odour having had 2 training sessions compared to 1 training session is:

$$\frac{\text{probability of detecting target odour after 2 training sessions}}{\text{probability of detecting target odour after 1 training session}} = e^{\beta_{10}}$$

where β_{10} is the estimate of β_{10} after fitting (1) to the data. A formula was also used for computing a confidence interval for the odds ratio based on the standard errors attached to each of the β_{js} in (1). Since the number of washes is a quantitative variable result we have for $k = 1, 2, 3, 4$ and 5 washes:

odds ratio of detecting target odour without any washes

$$\text{compared with after } k \text{ washes} = e^{k\beta}$$

Thus, a 95% confidence interval (C.I.) was calculated to accompany the odds ratio and gives the range of minimum (lower) and maximum (upper) that the odds ratio might be given 95% confidence. If the C.I. (range between lower and upper) were to cross the value of 1 than this would indicate that the effect on locating cotton with blood was weak and considered not statistically significant, whereas if the C.I. is greater than 1 this would indicate a strong effect on locating cotton with blood and be considered statistically significant.

2.3. Presumptive chemical testing (luminol)

For each set of unwashed and washed samples a subset of swatches were sprayed with luminol solution in order to presumptively test for the presence of blood (refer to Table 1). Along with the bloodstained cotton swatches a washed negative control was tested to make sure no cross-contamination had occurred during the washing process or that there was no false positive luminescence occurring from the washing powder. An unwashed negative control was also sprayed with luminol to test the baseline luminescence of the cotton material, as well as a cotton swatch containing fresh blood to represent an unwashed positive control.

The luminol solution was prepared fresh for each washing cycle and sprayed onto the swatches within 2 h of preparation. A 250 mL volume of solution was prepared from 12.5 g of sodium carbonate (Sigma-Aldrich, Castle Hill, NSW, Australia) dissolved in distilled water and mixed with 0.25 g of luminol powder (Sigma-Aldrich). Immediately after, 0.75 mL hydrogen peroxide (100 vol/30%, Sigma-Aldrich) was added to this solution. After the swatches were sprayed with luminol they were placed in a dark room for visualisation and photographed (Nikon D5200 24.1 Megapixel Digital Single-lens Reflex Camera) for documentation.

2.4. Odour profiling

Samples prepared for odour profiling were sealed prior to analysis in individual 250 mL aluminium tins (Morris McMahon & Co Pty Ltd, Sydney, NSW, Australia) to allow the headspace to accumulate and prevent contamination from the environment. The aluminium tins also replicated the current scenting methods employed by local law enforcement for the training of cadaver-detection and blood-detection dogs. All tins used in this study were cleaned prior to sample preparation by washing with hot water and non-phosphate detergent, and rinsing several times with de-ionised water and ultra-pure water prior to being baked in an oven for 1 h at 110 °C to remove potential volatile contaminants.

Unwashed and washed samples were not able to be analysed in one day due to lengthy manual sample extraction and analysis times and thus were staggered across 6 days with one set of washed samples and controls analysed per day. A single day of analysis included five replicates of cotton swatches containing blood (e.g. unwashed or washed 1–5 times), one cotton control which had been washed for the same number of cycles as the bloodstained swatch being analysed, and one cotton control which had not been washed. The odour profiling method used in this study included headspace SPME coupled with GC×GC-TOFMS, based on previous work conducted by the authors [22].

2.4.1. VOC sample collection

Headspace SPME sampling was performed using a previously optimised method [22]. A 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fused silica fibre (24-gauge; Supelco, Bellefonte, PA, USA) was inserted through a hole punctured into the aluminium tin lid containing the cotton swatch and exposed for 45 min at 40 °C using a sand bath for headspace extraction. Prior to sample exposure the fibre was exposed to an internal standard of 100 ppm bromobenzene (GC grade; Sigma-Aldrich, Castle Hill, NSW, Australia), prepared in methanol (HPLC grade; Sigma-Aldrich) using an in-fibre internal standardisation method [22]. Along with an internal standard, an alkane standard was also run once during each sampling day to highlight potential shifts in retention times from interday sampling.

2.4.2. GC×GC-TOFMS analysis

After VOC extraction, GC×GC-TOFMS analysis was performed according to a previously optimised and published procedure [22] using a LECO Pegasus 4D GC×GC-TOFMS system (LECO, Castle Hill, NSW, Australia) containing an Rxi[®]-624Sil MS (30 m × 0.250 mm inner diameter, 1.40 µm film thickness; Restek Corporation, Bellefonte, PA, USA) first dimension (¹D) column and a Stabilwax[®] (2 m × 0.250 mm inner diameter, 0.50 mm film thickness; Restek Corporation) second dimension (²D) column.

2.4.3. Data processing

Data processing was performed using ChromaTOF[®] (version 4.51.6.0; LECO). Baseline tracking with an 80% offset was performed with automatic baseline smoothing. An expected peak width of 15 s in the ¹D and 0.30 s in the ²D was used. A signal-to-noise ratio (S/N) of 250 for the base peak and 20 for the sub-peaks was set with a minimum match >800 for analyte identification. Library matches were generated from the 2011 National Institute of Standards and Technology (NIST) mass spectral library database.

The Statistical Compare software feature within ChromaTOF[®] (LECO) was used for peak alignment and normalisation to the internal standard using unique masses. Statistical Compare was performed using two methods. The first method included both washed and unwashed samples sorted into four classes (i.e. unwashed positive controls, unwashed negative controls, washed blood, washed negative controls) with compounds identified and included in the peak table if present in either at least 4 out of the total 40 samples or in 50% of the samples within a class. The second method included washed samples only sorted into two classes (i.e. washed blood and washed negative controls) with compounds identified and included in the peak table if present in either at least 4 out of the total 30 samples or in 50% of the samples within a class. Peak re-searching during alignment was performed at a S/N of 20.

A Fisher ratio was calculated using the Statistical Compare software feature to highlight compounds with the highest between-class variance. Compounds with Fisher ratios above the critical F value (F_{crit} – calculated using the F -distribution) of 2.89 or 4.21

(for each method respectively) were considered significant and filtered from the remaining compounds in the peak table for further statistical comparison. These compounds were exported as a .csv file and further processed in Microsoft Excel to manually remove chromatographic and internal standard artefacts. The unwashed blood samples analysed in this study were used for comparison purposes to identify blood-related compounds. These compounds were matched against previously reported blood VOCs found in previous research using this method [22] and used for tracking persistence in the washed samples.

Multivariate statistical analysis was carried out using principal component analysis (PCA) in The Unscrambler[®] X (version 10.3; CAMO Software, Oslo, Norway) to identify trends and group data using scores and loadings plots. Data pre-processing steps performed in The Unscrambler[®] X prior to PCA included mean centering, variance scaling, and unit vector normalisation which have been demonstrated to be beneficial for multivariate VOC analyses [24]. The data was verified to contain no outliers by implementing the Hotelling's T^2 95% confidence limit.

3. Results and discussion

3.1. Blood-detection and cadaver-detection dog trials

The blood-detection and cadaver-detection dogs utilised in this study had a range of training experience and represented various breeds and ages (refer to Table 2); thus the response rates recorded are organised by each team individually in order to account for the

potential influence of these factors, as well as compared as a whole group to identify overall trends in their general ability to locate latent blood.

The overall recorded false alerts collected in this study, as well as calculated sensitivity and specificity are summarised in Table 3. Sensitivity and specificity assist in evaluating the dog's accuracy to locate blood, whereby sensitivity represents the proportion of positive alerts that correctly identified cotton containing blood (true positive rate – the number of true positive alerts divided by the combined number of true positive and false negative alerts) and specificity represents the proportion of negative alerts that correctly identified when cotton did not contain blood (true negative rate – the number of true negative alerts divided by the combined number of true negative and false positive alerts).

Table 3 demonstrates a clear efficiency difference between each of the teams incorporated in this study, with some displaying higher correct alerts, sensitivity and specificity for both the unwashed and washed cotton with blood (most evident for teams A, B and E). For the unwashed cotton with blood, the sensitivity was calculated to range between 93% and 100%, and the specificity between 88% and 99% for all teams. For the washed cotton with blood, there was a much larger range calculated, with some teams having no calculated sensitivity or specificity for the more frequently washed cotton with blood (i.e. washes 3–5).

Looking at the teams as a whole, due to the low accuracy for many teams to locate the washed cotton with blood the average sensitivity ranged between 41 and 96%, and specificity ranged between 81 and 95%. This indicates a greater proficiency of these

Table 3
Summary of recorded alerts for the limit of detection trials conducted with the blood-detection and cadaver-detection dogs in this study.

	Cotton Swatches (With/Without Blood)	Recorded False Alerts (%)		Evaluation (%)	
		False Positive	False Negative	Sensitivity	Specificity
Team A	Unwashed	9	0	100	92
	Wash 1	17	0	100	86
	Wash 2	11	0	100	90
	Wash 3	6	33	67	92
	Wash 4	0	50	50	100
	Wash 5	6	0	100	95
Team B	Unwashed	4	7	93	96
	Wash 1	11	11	89	89
	Wash 2	7	0	100	93
	Wash 3	0	22	78	100
	Wash 4	4	11	89	96
	Wash 5	7	33	67	90
Team C	Unwashed	1	6	94	99
	Wash 1	6	50	50	90
	Wash 2	6	67	33	86
	Wash 3	6	100	0	0
	Wash 4	6	83	17	75
	Wash 5	11	83	17	60
Team D	Unwashed	1	0	100	99
	Wash 1	6	50	50	90
	Wash 2	6	83	17	75
	Wash 3	6	100	0	0
	Wash 4	6	83	17	75
	Wash 5	6	100	0	0
Team E	Unwashed	13	6	94	88
	Wash 1	17	50	50	75
	Wash 2	6	67	33	86
	Wash 3	22	17	83	79
	Wash 4	33	67	33	50
	Wash 5	17	67	33	67
Averages of Teams A-E	Unwashed	6	4	96	95
	Wash 1	11	32	68	86
	Wash 2	7	43	57	89
	Wash 3	8	54	46	85
	Wash 4	10	59	41	81
	Wash 5	9	57	43	82

dogs to identify when blood is not present (high true negative rate) at a baseline level. Similarly, the overall average recorded false positives were considerably low (9%) but much higher for the false negative alerts (42%). Observing the false positive responses in detail (when the dog incorrectly alerted to washed and unwashed cotton without blood) can assist in identifying control odours that present a potential issue for these scent-detection dogs in locating their target odour. Overall, the percentage of false positive alerts does not appear to be associated with any cotton control specifically.

Observing the percentage of correct alerts recorded and averaged for all three training sessions (Fig. 2), it is apparent that as the number of washes increases the percentage of correct alerts decreases, with varying responses recorded for each team. After one wash, teams C, D and E were able to locate 50% of their target samples, and after two washes these teams located approximately one third of the target samples. After three washes, the teams' responses appear to be erratic in locating the washed blood. It is hypothesised that as the blood is washed through more cycles, the VOCs that the dogs use to locate the odour becomes diminished making it more difficult to identify from the background odours of the cotton material.

By presenting the data with all teams represented individually, the variation in performance across the dogs is apparent with some teams having a greater percentage of correct alerts across more of the washes, as discussed previously. Teams A and B performed the strongest across the training sessions, locating blood washed up to five times. Team E also located blood washed up to five times; however, this team did not have consistent true positive alerts across the washes. This is demonstrated by their low accuracy in locating blood washed two, four and five times, but having a high accuracy for blood washed three times and the unwashed blood. Teams C and D demonstrated a dramatic loss in efficiency in locating the blood when washed more than once, with a detection limit of up to two washes, albeit with very low accuracy. It is hypothesised that reinforcement on the washed blood would be required in order to increase their sensitivity to multiple washes, and potentially more training and exposure to the diluted odour would improve their accuracy.

The odds of detection for unwashed blood on cotton (zero washes) compared to five washes displayed strong statistical significance (C.I. much greater than 1) which confirm the findings in Fig. 2, with the dogs having greater odds of finding unwashed

cotton with blood than blood washed five times on cotton (Table 4). As the number of washes increased, the odds ratio values were much greater in favour of the unwashed cotton with blood, and thus the probability of detection also decreases, demonstrating that the dogs are more likely to locate blood on cotton which has not been washed than blood on cotton washed. Whilst relatively low in value, the zero washes compared with one wash still demonstrates statistical significance indicating a low probability for these dogs to locate blood on cotton washed even just once, and thus more training would be required to improve their probability of locating latent blood.

When comparing the cadaver-detection and blood-detection dogs used in this study, the probability of detection was not shown to be statistically significant for detecting unwashed blood on cotton (zero washes) and therefore there was no performance difference observed between the two sets of teams. Conversely, although only one blood-detection dog was available for this study (team D), the majority of the other teams A, B, C and E (all cadaver-detection dogs) performed slightly better with the washed blood samples, with a statistically significant interaction effect observed. These results could relate to the training experience of the cadaver-detection dogs with multiple training aids (i.e. blood, bone, decomposition fluid etc.) and could indicate a difference between the two detector dog types. It is acknowledged that this was only a baseline study with only a small sample size of scent-detection dogs, and thus more trials would need to be conducted on a larger number of both blood-detection and cadaver-detection dogs to comment on any significant trend for the general population of blood-detection dogs.

Due to the operational commitments of the cadaver-detection and blood-detection dogs involved in this study, the majority of teams (A, C, D, and E) were only available for two of the three training sessions, with irregular attendance sometimes resulting in large gaps between each team's training sessions. However, by observing each team individually by the correct alerts at their relative first and second exposure (rather than by training dates), in some instances there is an improvement in their accuracy with repeated exposure (Fig. 3). The calculated probabilities confirm this, with the odds of detection greater after the second training compared to the first training session (but not as high as seen with the other effects calculated) and shown to be statistically significant.

Team A improved their ability to locate samples subjected to a higher number of washes by their second training session, with

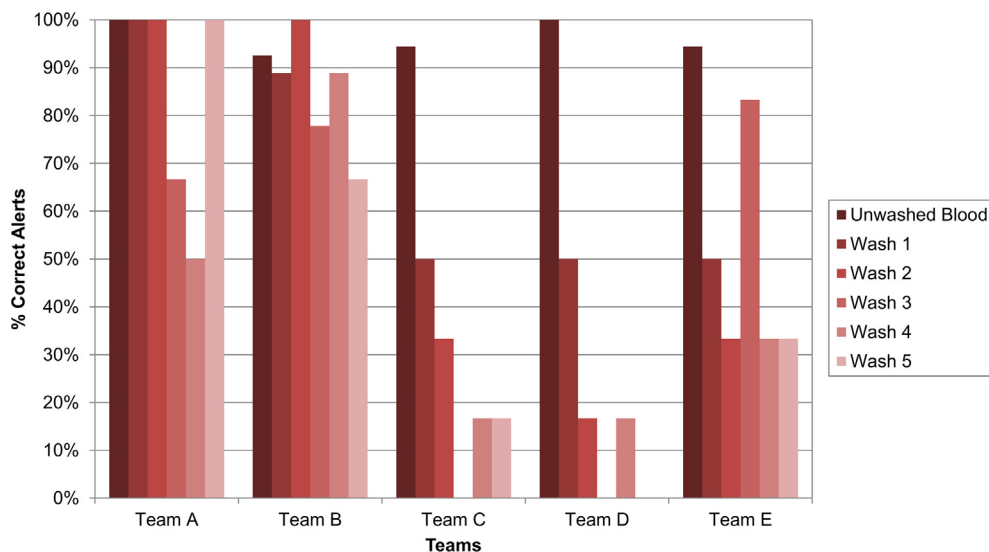


Fig. 2. Overall percentage of correct alerts recorded for cadaver-detection and blood-detection dogs when exposed to unwashed and washed blood on cotton.

Table 4

Summary of probabilities calculated for the cadaver-detection and blood-detection dogs to detect the presence of blood given the effect of washing, training and type of detection.

Calculated Effect		Odds Ratio (95% C.I.)		
		Lower	Estimate	Upper
Number of washes	0 washes compared with 5 washes	77.9	9130	1.07×10^6
	0 washes compared with 4 washes	32.6	1470.0	6.66×10^4
	0 washes compared with 3 washes	13.6	238.0	4140
	0 washes compared with 2 washes	5.71	38.40	258.00
	0 washes compared with 1 wash	2.39	6.20	16.10
Type of detection dog	Teams A, B, C & E vs. Team D when there are zero washes	0.0936	0.466	2.32
	Teams A, B, C & E vs. Team D when there are 5 washes	7.1	293.0	1.21×10^4
Number of training sessions	2nd training session compared with 1st training session	1.27	2.78	6.07
	3rd training session compared with 1st training session [*]	0.464	1.79	6.91
	3rd training session compared with 2nd training session [†]	0.156	0.644	2.65

^{*} Note: only one dog completed three training sessions.
[†] Values reported are specific to this study only.

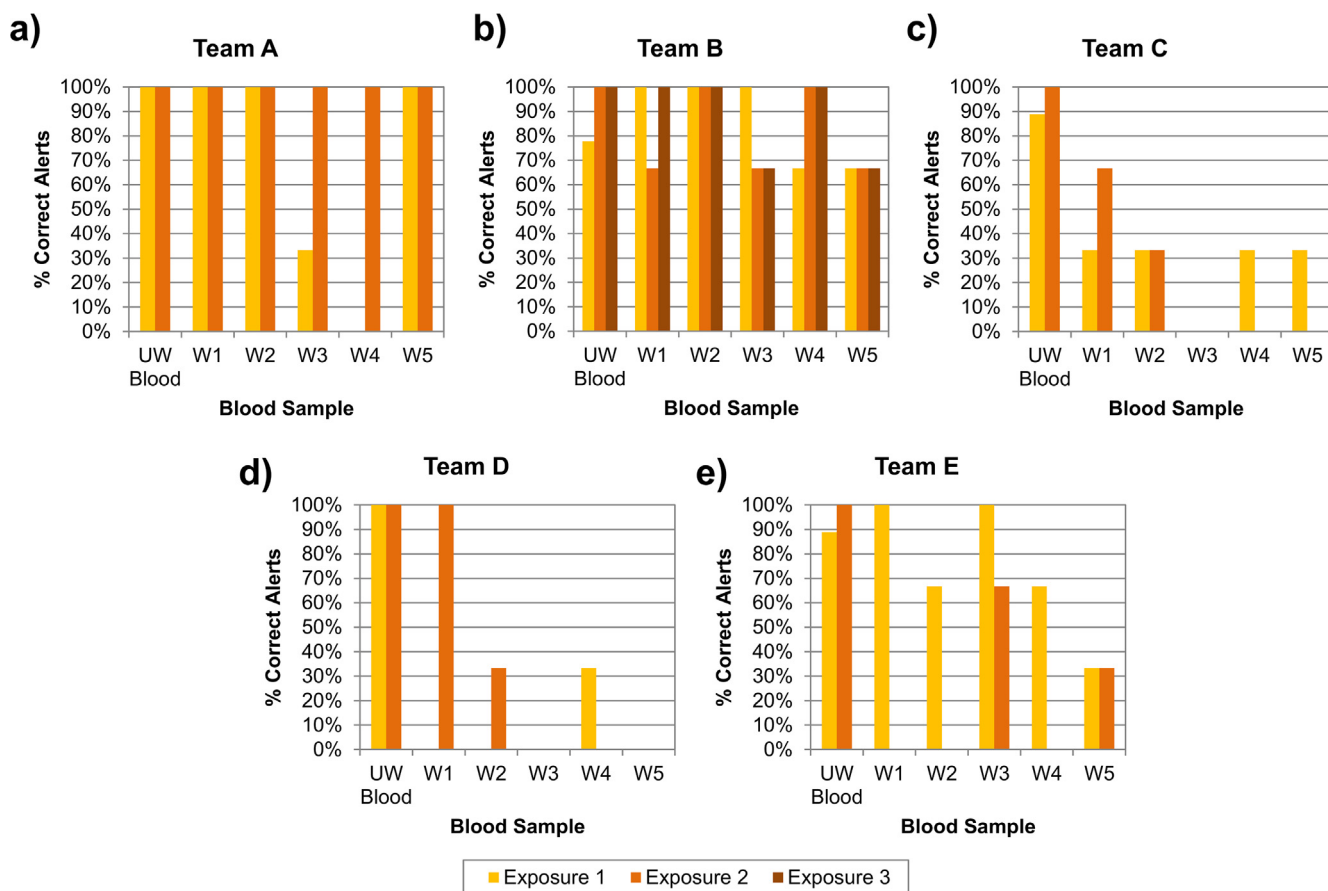


Fig. 3. Percentage of correct alerts recorded for unwashed (UW) and washed (W) blood (washed 1–5 times) for a) team A, b) team B, c) team C, d) team D and e) team E across the training sessions they were present at over the duration of this study (exposure 1–3).

100% accuracy (Fig. 3a). Team C, while able to locate blood washed up to five times in the first training session, was also able to locate blood washed once with greater accuracy by the second training session (Fig. 3c). Team D could not locate most of the washed target samples initially at their first training session but located blood washed up to two times by their second training session (Fig. 3d). Although team D did initially locate some of the blood samples washed up to four times at the first training session, many of the lesser washed samples were missed which suggests that they only located the blood washed four times by chance.

Team E did not appear to improve consistently like the other teams across the two training sessions; however, this team was still able to maintain the ability to locate blood even after five

washes (Fig. 3e). At the second training session, it was noted that this team gave several partial alerts (i.e. head flicks) and did show interest to the missed washed blood samples but did not complete a full alert for recording. This team had also performed a larger number of false positive responses at the second training session, more than double recorded for his first training session. Team E was the youngest cadaver-detection dog with the fewest years of operational experience, which is important to consider when evaluating his responses.

For team B, the percentage of correct alerts recorded for each exposure to the washed and unwashed samples (Fig. 3b) demonstrated a slight improvement in consistency in locating the more heavily washed samples (washed two and more times). With reg-

ular training and exposure to this set of washed blood samples, this team maintained a consistent accuracy in locating the target blood samples across the duration of this study. Team B was the only team present for all three training sessions; however, it was not evidence enough with the probabilities to say that the third training improved their odds of detection. Given that there was only one dog that completed the three training sessions, a trial with more dogs present at all training sessions would need to be studied to confirm any associated influence of this effect. Note that this study intended to use a low number of training sessions to specifically test the dogs' baseline abilities without further training.

The results of this study demonstrate the potential of blood-detection and cadaver-detection dogs in locating washed blood, with some dogs at a baseline level able to confidently detect blood washed up to five times. By understanding the detection limits of these dogs, their responses in the field can be validated even when the evidence is not visible. This is significant when considering controversial cases where blood-detection and cadaver-detection dogs have signalled the presence of blood at a crime scene without substantial confirmation of the evidence [11,25]. The use of these dogs as a screening tool for latent blood would ideally represent a complementary technique for chemical presumptive tests but would not replace further confirmatory tests as part of an investigation. It is noted however, that chemical confirmatory tests are

often less sensitive than chemical presumptive tests, and more expensive to run. Thus, having confidence in the alerts produced by these dogs can assist investigators in qualifying the use of subsequent costly tests.

3.2. Presumptive chemical testing (luminol)

A luminol solution was prepared fresh on the day of analysis and visualised in a dark room minutes after spraying, where only qualitative luminescence was recorded for this study. The aim was to determine whether the luminol was effective in detecting latent blood after several cycles of washing through visual luminescence.

Before the washing cycles commenced, five replicates of unwashed positive controls were prepared and visualised with a single replicate of unwashed negative control to observe the degree of luminescence in the presence of blood and whether the cotton swatches exhibited any background luminescence (Fig. 4a). It was determined that the unwashed cotton swatches did not display any natural luminescence, and thus would not enhance the luminescence when blood was present. A strong luminescence was visualised in reaction to the unwashed positive controls with dark spots apparent in areas of high blood volume.

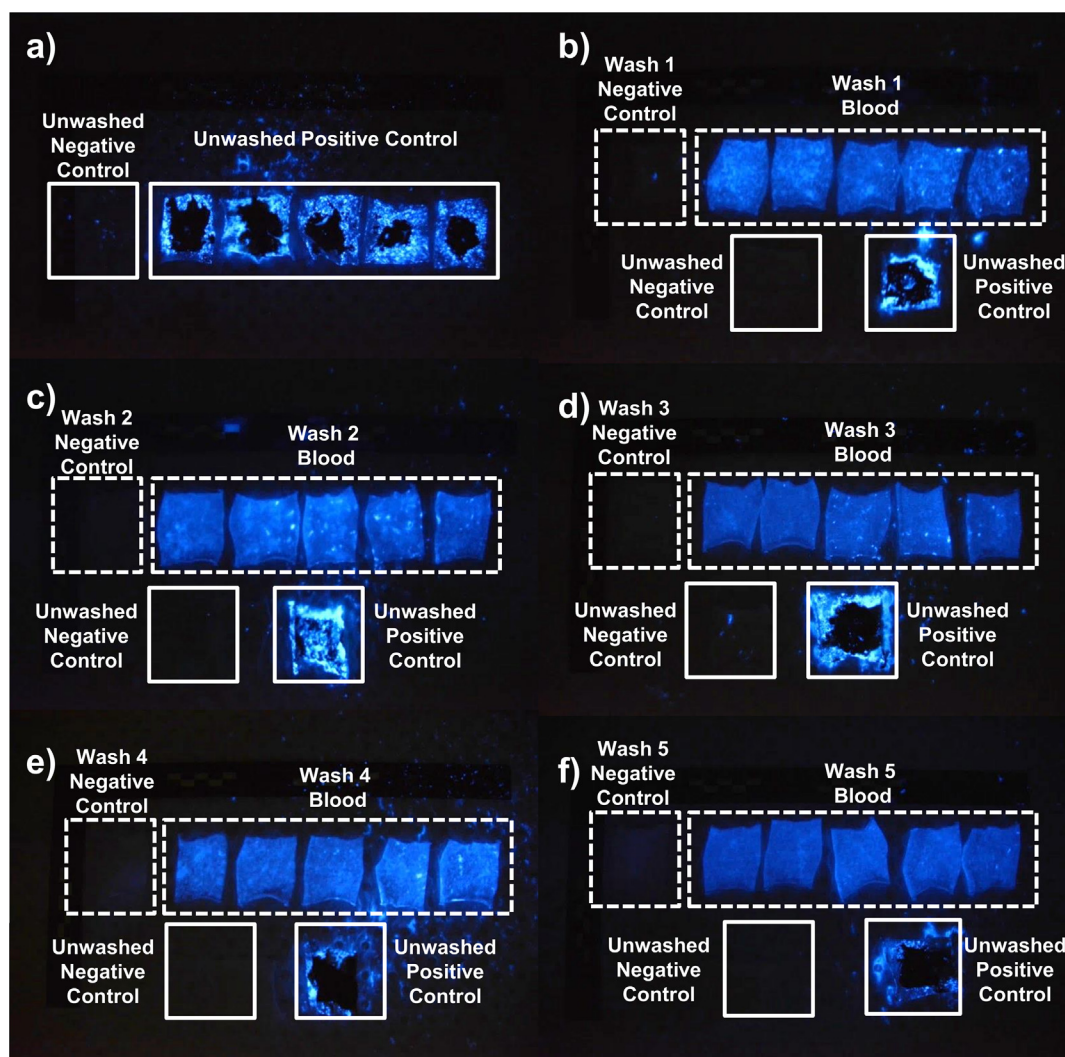


Fig. 4. Luminol reaction observed on blood and control swatches for a) unwashed positive control; and blood after b) 1 wash, c) 2 washes, d) 3 washes, e) 4 washes and f) 5 washes.

The results demonstrated that luminol was effective in detecting blood washed up to five times on cotton swatches (Fig. 4b–f), with luminescence visually evident across all replicates. The unwashed cotton swatch, washed cotton swatches without blood (i.e. negative controls) and unwashed cotton swatches with fresh blood (i.e. positive controls) were incorporated for visual comparison and to identify false positives or false negatives. The washed negative control did not exhibit any luminescence except for wash five, which showed a very weak luminescence compared to the negative control (Fig. 4f). This indicates that the laundry powder does not contain any substantial interfering agents in reaction to luminol to create false positive results. Similarly, the unwashed positive control always showed luminescence, which confirmed that the luminol solution was working as expected.

The incorporation of the luminol presumptive chemical test in this study represented the most common reagent employed by crime scene officers due to its reported high sensitivity for blood, and compatibility for the extraction of DNA [8,9]. The effectiveness of the luminol on all of the washed samples confirmed that the presence of blood is still detectable even when washed up to five times. The results in this study indicate that the use of luminol or scent-detection dogs as a screening tool for latent blood at crime scene searches are both viable options. Thus, if an operational scenario is unsuitable for luminol, such as large outdoor areas or on surfaces prone to false positive results, blood-detection and cadaver-detection dogs could be used as an alternative technique for locating blood with similar efficiency and sensitivity. Luminol has been reported to have the sensitivity to detect blood diluted with water as low as 1:5,000,000 on blood soaked cotton [26], but more commonly is reported to have a detection limit of 1:100,000 with recoverable DNA [8,27]. If the blood-detection and cadaver-detection dogs improve their sensitivity to the reported levels of luminol, their operational use would be highly

valuable to secure evidence with the potential for DNA extraction and subsequent investigative leads.

3.3. Odour profiling

The ability of the blood-detection and cadaver-detection dogs to detect blood after as many as five washes in this study indicates that some portion of the blood odour profile remains intact throughout the washing cycles. In order to evaluate the blood odour profile from the background odour of the cotton or laundry powder, the unwashed blood, washed blood and negative control samples were tested using the highly sensitive analytical instrumentation: GC×GC-TOFMS. GC×GC has a reported 10-fold increase in sensitivity compared to conventional one-dimensional-GC (1D-GC), with detection limits in the low parts-per-trillion (ppt) [28,29], and thus was deemed the most appropriate analytical instrumentation to apply to this study.

Initially, the chromatographic outputs produced by GC×GC-TOFMS visually showed a distinct difference in profile between the unwashed negative control (Fig. 5a) and the unwashed positive control (Fig. 5b), with a greater number of compounds detected for the unwashed positive control. Blood washed once (and similarly observed for washes two to five) with laundry powder produced a more complex chromatogram to that of the unwashed positive control (Fig. 5d and b, respectively), with more VOCs apparent across the chromatogram. Conversely, the washed negative control washed once (Fig. 5c) compared to the blood on cotton washed once (Fig. 5d) produced chromatograms that were visually indistinguishable, and this trend remained consistent across all washes. It appears that the washing procedure added more volatile components to the cotton material, such that odourous components of the laundry powder were becoming trapped within the cotton

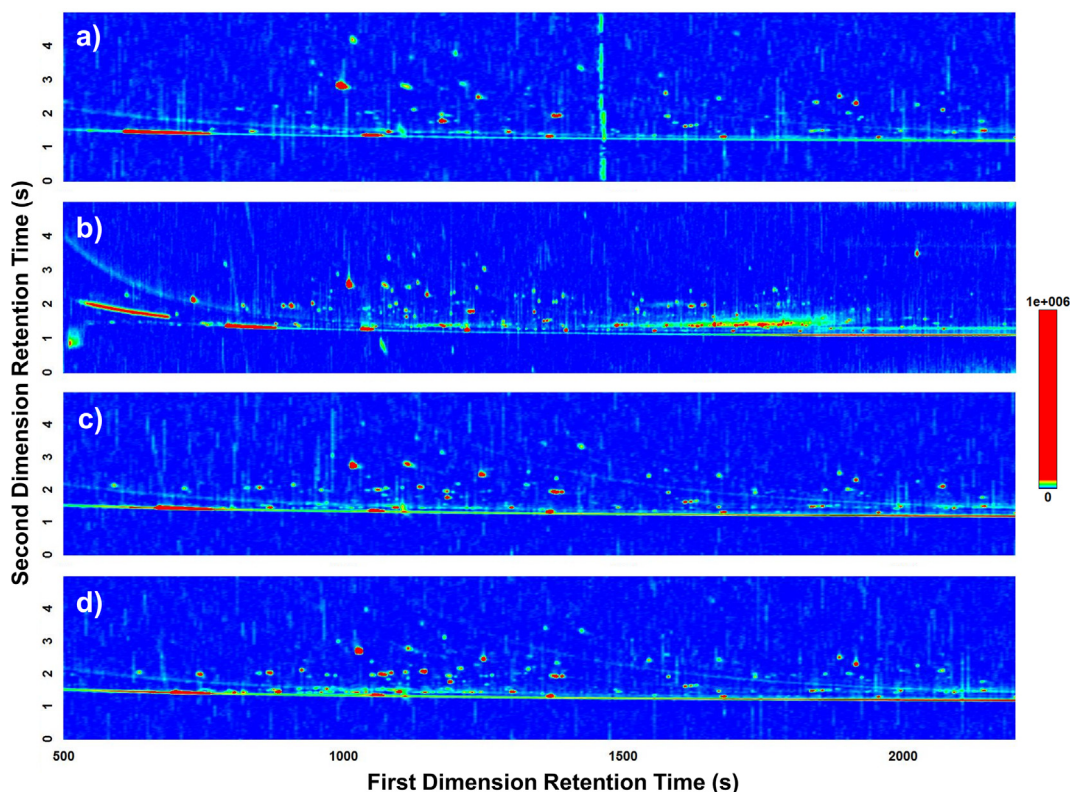


Fig. 5. GC×GC-TOFMS total ion current (TIC) contour plots (500–2200 s) of a) unwashed negative control, b) unwashed positive control, c) washed negative control washed once and d) cotton with blood washed once, showing increase in profile complexity after first wash.

fibres, while the blood odour profile was visually absent suggesting that it may have been removed by washing.

Determining that an odour profile from the laundry powder is retained after washing is an important training consideration for blood-detection and cadaver-detection dogs. In order to reduce potential false positives occurring, these dogs should be trained off the background odour of the washed cotton by incorporating washed negative controls within the training procedure. As identified in the dog trials of this study, the blood-detection and cadaver-detection dogs more often falsely alerted on the washed negative controls, which indicated that there was a common odour between the washed cotton without blood and washed cotton with blood that the dogs could not differentiate.

The low overall occurrence of false positive responses compared to the percentage of correct alerts would indicate that the olfactory system of the dogs in this study was able to distinguish odour differences between the washed blood and negative controls that are not visually apparent with the GC×GC chromatograms. The data collected from the GC×GC-TOFMS was analysed using PCA to determine whether the VOC profile of the washed and unwashed blood could be separated from that of the washed and unwashed negative controls. PCA assists in visualising differences in the GC×GC data by reducing the data dimensionality. Fig. 6a demonstrated that the washed and unwashed positive controls were clearly separated on the first principal component (PC-1) based on their VOC profile, which accounted for 33% of the variation

within the dataset. However, within these clusters, the blood and negative controls could not be distinguished and overlapped considerably in the PCA plot. Observing the dataset from a three-dimensional (3D) perspective using PC-1, the second principal component (PC-2) and the third principal component (PC-3) (Fig. 6b) allowed for the visualisation of separation between the unwashed positive control and unwashed negative controls, with 11% of the variation within the dataset accounted for on PC-3. This separation between the unwashed positive control and unwashed negative controls was expected based on the visual comparison of the chromatograms (Fig. 5a and b). A large portion of the profile detected appeared to contribute to the washed blood and controls as displayed in the correlation loadings plot in Fig. 6c, with the majority of the discriminatory compounds dominating the bottom two quadrants (namely compounds 13–62). Since the chromatograms in Fig. 5 displayed a greater complexity for the washed blood and washed negative controls, this would indicate that these compounds could be attributed to the washing process (i.e. laundry powder). However, with little separation apparent between the washed negative controls and washed cotton with blood, it is difficult to determine whether these discriminatory compounds were related to the washed cotton or the latent blood odour.

Analysing the dataset with PCA was not effective in separating the VOC profiles of the washed blood samples from the washed negative controls, and this is likely because the variation between the unwashed and washed samples was so much larger than that

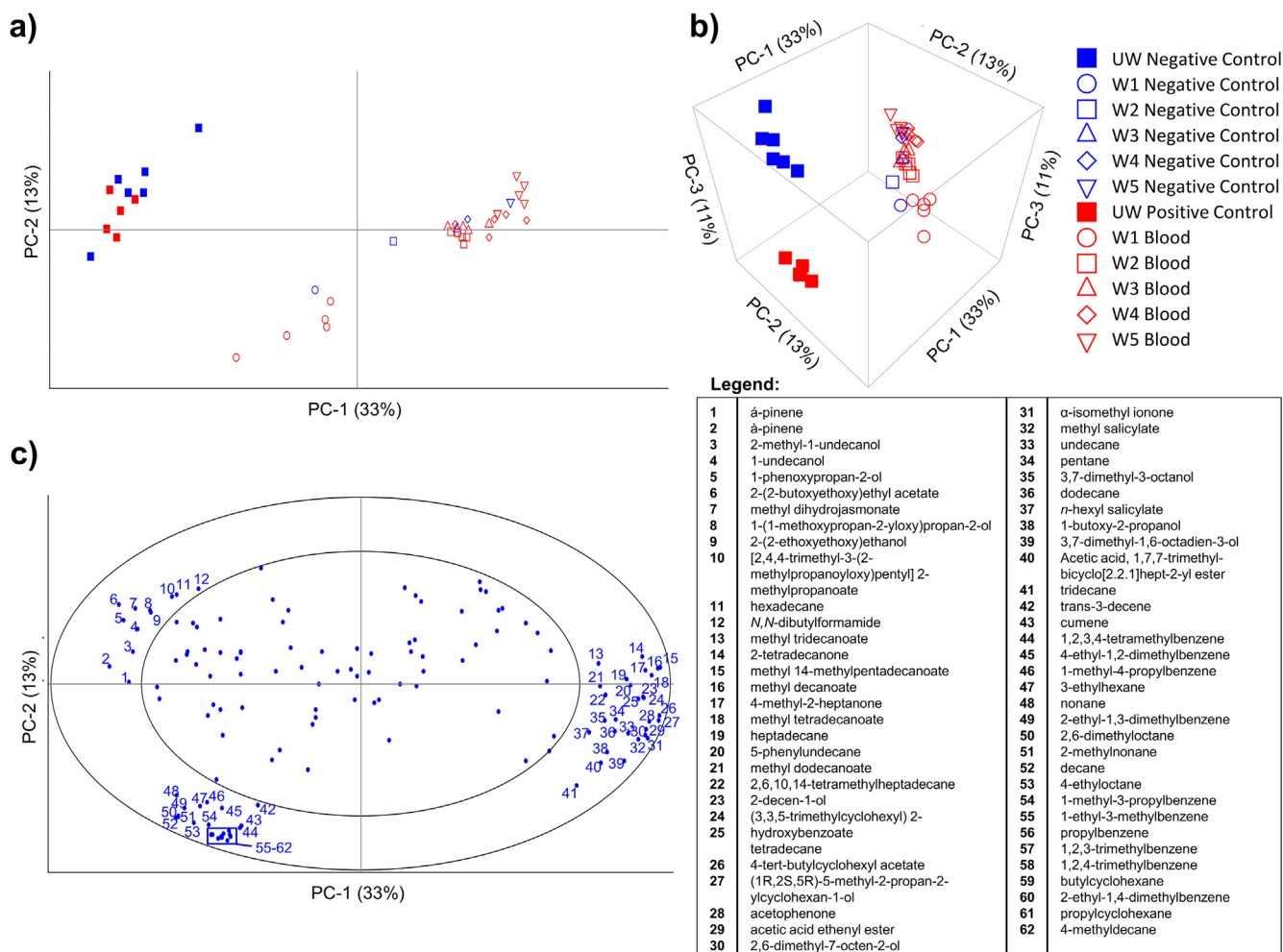


Fig. 6. PCA plots of compounds detected in comparison of unwashed (UW) and washed (washes 1–5: W1–W5) blood and controls: a) scores plot of PC-1 and PC-2, b) 3D scores plot of PC-1, PC-2 and PC-3 and c) correlation loadings plot of PC-1 and PC-2.

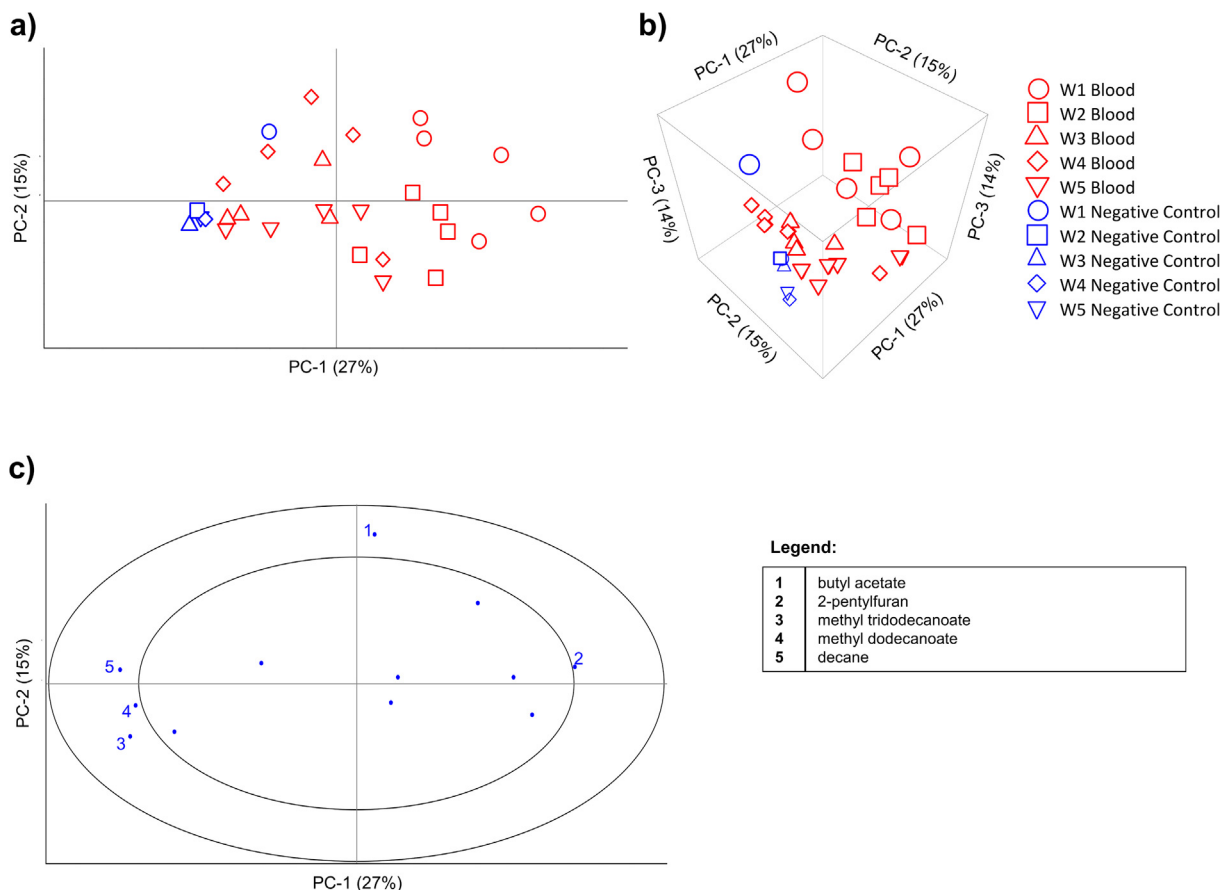


Fig. 7. PCA plots of compounds detected in comparison of washed (washes 1–5: W1–W5) blood and negative controls: a) scores plot of PC-1 and PC-2, b) 3D scores plot of PC-1, PC-2 and PC-3 and c) correlation loadings plot of PC-1 and PC-2.

between the washed blood and washed negative controls causing the washed samples to group tightly and skew the results. It was therefore decided to further investigate the odour profiles of the washed blood and washed negative controls separate from the unwashed controls to better visualise any subtle differences in the profiles. It is important to note here that only one washed negative control was analysed per wash cycle using GC×GC-TOFMS due to time restraints involved with the use of manual HS-SPME, and thus, it is recommended that future work incorporate more washed negative controls.

In a second Statistical Compare method, washed cotton with blood and washed negative controls were compared and further analysed with PCA. Fig. 7a and b demonstrate that the blood washed once and twice (wash 1 and wash 2 blood) could be separated from the washed negative controls on PC-1, which accounted for 27% of the variation within the dataset, and further separated on PC-3 which accounted for 14% of variation within the dataset. After Fisher ratio filtering the compounds detected in the washed blood and washed negative controls, there were only twelve compounds significant to the dataset, of which only five compounds were identified as highly discriminatory for the dataset, namely 2-pentylfuran, butyl acetate, decane, methyl dodecanoate, and methyl tridodecanoate, shown in Fig. 7c. Two of these compounds, 2-pentylfuran and butyl acetate, appear to be associated with the washed blood samples in the PCA plot, with 2-pentylfuran previously cited in the literature as a blood VOC [30–32]. The other three discriminatory compounds (decane, methyl dodecanoate, and methyl tridodecanoate) appear to be associated with the washed negative controls, with only decane being previously reported in the blood odour profile [31,33]. These compounds

could potentially be responsible for differentiating the washed blood samples from the washed negative controls and could represent the VOCs that the dogs recognise.

4. Conclusions

Blood-detection dogs and cadaver-detection dogs have been widely regarded as highly sensitive search tools, with their power of sensitivity anecdotally reported to locate minute traces of blood even after washing with detergents. However, to date, very few research studies have investigated these detection limits scientifically to validate anecdotal claims. This study aimed to investigate the sensitivity of blood-detection and cadaver-detection dogs to detect latent blood which had been washed up to five times with household laundry detergent, and compare their efficiency with current screening tools for crime scene searches and with highly sensitive analytical instrumentation.

It was determined that at a baseline level, blood-detection and cadaver-detection dogs are able to locate blood washed up to five times and can differentiate these from control odours. It was discovered that the efficiency to locate these latent odours was highly variable from dog to dog, with their personality, experience and temperament identified to be potential sources of influence. Due to the small number of scent-detection dogs available for this study, it is recommended that further research be conducted with a larger group of dogs in order to confirm the trends observed in this study. It was also observed over two training sessions, that the blood-detection and cadaver-detection dogs showed some improvement in their sensitivity and specificity to the washed

blood samples. This highlights the potential of regular training and repeated exposure to latent blood for training scent-detection dogs to lower limits of detection; however, more research is required to confirm this finding.

Comparing the detection limits of the dogs to a common presumptive test used in crime scene searches (i.e. luminol) determined that the blood-detection and cadaver-detection dogs could act as complementary search tools, with both the dogs and luminol demonstrating the ability to detect blood washed up to five times on cotton. However, the luminol was observed to perform more consistently compared to the baseline capabilities of the detection dogs that had no prior training with washed blood on cotton. This study incorporated a common household laundry detergent and did not study the effects of bleaching agents, and thus further studies are recommended to investigate bleach on the accuracy of the dogs to locate latent blood. It is well established that luminol can react with blood diluted as low as 1:500,000. Confirming that the scent-detection dogs can match this sensitivity to washed blood will validate their use for crime scene searches that are deemed inefficient and costly for regular presumptive chemical tests. The use of these dogs would assist in narrowing areas of interest for further processing, for example, with the potential to locate items of high evidentiary value that may have been previously overlooked.

Analysing the unwashed and washed blood and control samples using GC×GC-TOFMS provided an insight into the potential odour profile that the blood-detection and cadaver-detection dogs recognise as part of their scenting process, with only a few VOCs deemed significant for the odour profile of washed blood. GC×GC demonstrated a detection limit of up to two washes for blood samples, with samples subjected to three to five washes becoming difficult to distinguish from their respective washed negative controls based on VOC profiling. Current analytical techniques are not able to match the sensitivity of luminol or the scent-detection dogs observed in this study, but they provide confirmation that subtle odour differences are apparent between washed blood and washed negative controls that these dogs can perceive. However, ultimately investigators must determine whether lowering the detection limits of their dogs to dilute blood is suitable for their operational aims, and recognise that further analytical confirmatory tests may not be able to match the sensitivity of the dogs.

This study was able to track the odour of blood after multiple washes using GC×GC as an analytical tool and provide potential chemistry behind blood-detection and cadaver-detection dog alerts operationally to latent blood on cotton clothing. By establishing the detection limits of these dogs at a baseline level, handlers can be confident of their alerts in the field, and thus provide law enforcement with valuable information for building stronger investigations. Regular training with latent blood samples is recommended in order to provide the dogs with an accurate representation of the odour profiles they may encounter operationally and to improve their sensitivity in cases of concealment.

Acknowledgements

The authors wish to thank the external law enforcement agency for providing the cadaver-detection and blood-detection dogs for this study, and for giving their time, support and advice in our on-going research. The authors would also like to thank all of the research members for their hard work and patience in setting up the dog trials: Barea Chilcote, Darshil Patel and Nicole L. Cattarossi. Laboratory technical staff, Dr Ronald Shimmon and Dr Regina V. Taudte, are recognised for their ongoing support. The Australian Research Council (ARC), the University of Technology Sydney (UTS), and the Australian Government Research Training Program

Scholarship (RTP) are acknowledged for providing financial support for this research.

Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.forc.2018.05.002>.

References

- [1] T.W. Adair, R.L. Shaw, Enhancement of bloodstains on washed clothing using luminol and LCV reagents, *J. Bloodstain Pattern Anal.* 2005 (December 2005) 4–10.
- [2] A. Castelló, F. Francés, F. Verdú, Bleach interference in forensic luminol tests on porous surfaces: more about the drying time effect, *Talanta* 77 (2009) 1555–1557.
- [3] K.A. Harris, C.R. Thacker, D. Ballard, D.S. Court, The effect of cleaning agents on the DNA analysis of blood stains deposited on different substrates, *Int. Congr. Ser.* 1288 (2006) 589–591.
- [4] L. Gefrides, K. Welch, Forensic biology: serology and DNA, in: A. Mozayani, C. Noziglia (Eds.), *The Forensic Laboratory Handbook Procedures and Practice*, Humana Press, 2011, pp. 15–50.
- [5] R. Saferstein, *Criminalistics: An Introduction to Forensic Science*, tenth ed., Pearson Education, Upper Saddle River, NJ, USA, 2011.
- [6] M. Vennemann, G. Scott, L. Curran, F. Bittner, S.S. Tobe, Sensitivity and specificity of presumptive tests for blood, saliva and semen, *Forensic Sci. Med. Pathol.* 10 (2014) 69–75.
- [7] F. Barni, S.W. Lewis, A. Berti, G.M. Miskelly, G. Lago, Forensic application of the luminol reaction as a presumptive test for latent blood detection, *Talanta* 72 (2007) 896–913.
- [8] S.S. Tobe, N. Watson, N.N. Daeid, Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA, *J. Forensic Sci.* 52 (2007) 102–109.
- [9] J.I. Creamer, T.I. Quickenden, L.B. Crichton, P. Robertson, R.A. Ruhayel, Attempted cleaning of bloodstains and its effect on the forensic luminol test, *Luminescence* 20 (2005) 411–413.
- [10] M. Cox, Effect of fabric washing on the presumptive identification of bloodstains, *J. Forensic Sci.* 35 (1990) 1335–1341.
- [11] N. Bunyan, *The Police Dog that Earns More than Her Chief Constable*, The Telegraph, United Kingdom, 2005.
- [12] M.M. Cerreta, K.G. Furton, An assessment of detection canine alerts using flowers that release methyl benzoate, the cocaine odorant, and an evaluation of their behavior in terms of the VOCs produced, *Forensic Sci. Int.* 251 (2015) 107–114.
- [13] L. Lazarowski, D.C. Dorman, Explosives detection by military working dogs: olfactory generalization from components to mixtures, *Appl. Anim. Behav. Sci.* 151 (2014) 84–93.
- [14] M.B. Alexander, T.K. Hodges, J. Bytheway, J.A. Aitkenhead-Peterson, Application of soil in forensic science: residual odor and HRD dogs, *Forensic Sci. Int.* 249 (2015) 304–313.
- [15] M.B. Alexander, T.K. Hodges, D.J. Wescott, J.A. Aitkenhead-Peterson, The effects of soil texture on the ability of human remains detection dogs to detect buried human remains, *J. Forensic Sci.* 61 (2016) 649–655.
- [16] I. Riezzo, M. Neri, M. Rendine, A. Bellifemina, S. Cantatore, C. Fiore, E. Turillazzi, Cadaver dogs: unscientific myth or reliable biological devices?, *Forensic Sci. Int.* 244 (2014) 213–221.
- [17] A.G. Skalleberg, M.M. Bouzga, Detecting and collecting traces of semen and blood from outdoor crime scenes using crime scene dogs and presumptive tests, *Forensic Sci. Int.* 264 (2016) 146–152.
- [18] A. Schoon, A comparison between canine detection of blood residue and some blood presumptive tests, *J. Forensic Identif.* 63 (2013) 255–262.
- [19] R.C. Buis, L. Rust, K.D. Nizio, T. Rai, B.H. Stuart, S.L. Forbes, Investigating the sensitivity of cadaver-detection dogs to decomposition fluid, *J. Forensic Identif.* 65 (2015) 985–997.
- [20] M. Bensafi, C. Zelano, B. Johnson, J. Mainland, R. Khan, N. Sobel, Olfaction: from sniff to percept, in: M.S. Gazzaniga, G.R. Mangun (Eds.), *The Cognitive Neurosciences*, MIT Press, Massachusetts, 2014.
- [21] C. Angle, L.P. Waggoner, A. Ferrando, P. Haney, T. Passler, Canine detection of the volatilome: a review of implications for pathogen and disease detection, *Front. Vet. Sci.* 3 (2016) 47.
- [22] L. Rust, K.D. Nizio, S.L. Forbes, The influence of ageing and surface type on the odour profile of blood-detection dog training aids, *Anal. Bioanal. Chem.* 408 (2016) 6349–6360.
- [23] B. Rosner, *Fundamentals of Biostatistics*, fourth ed., Duxbury Press, Belmont, Massachusetts, USA, 1995.

- [24] R.A. van den Berg, H.C. Hoefsloot, J.A. Westerhuis, A.K. Smilde, M.J. van der Werf, Centering, scaling, and transformations: improving the biological information content of metabolomics data, *BMC Genomics* 7 (2006) 142.
- [25] R. Edwards, Madeleine Sniffer Dogs Detect Scent of Body, *The Telegraph*, United Kingdom, 2007.
- [26] J.L. Webb, J.I. Creamer, T.I. Quickenden, A comparison of the presumptive luminol test for blood with four non-chemiluminescent forensic techniques, *Luminescence* 21 (2006) 214–220.
- [27] B. Budowle, J.L. Leggitt, D.A. Defenbaugh, K.M. Keys, S. Malkiewicz, The presumptive reagent fluorescein for detection of dilute bloodstains and subsequent STR typing of recovered DNA, *J. Forensic Sci.* 45 (2000) 1090–1092.
- [28] Z. Liu, J. Phillips, Sensitivity and detection limit enhancement of gas chromatographic detection by thermal modulation, *J. Microcolumn Sep.* 6 (1994) 229–235.
- [29] R. Hites, Gas chromatography-mass spectrometry, in: F. Settle (Ed.), *Handbook of Instrumental Techniques for Analytical Chemistry*, Prentice-Hall Inc, Upper Saddle River, NJ, USA, 1997.
- [30] M. Kusano, E. Mendez, K.G. Furton, Comparison of the volatile organic compounds from different biological specimens for profiling potential, *J. Forensic Sci.* 58 (2013) 29–39.
- [31] S.L. Forbes, L. Rust, K.A. Perrault, K. Trebilcock, L.T. McGrath, Effect of age and storage conditions on the volatile organic compound profile of blood, *Forensic Sci. Med. Pathol.* 10 (2014) 570–582.
- [32] L.M. Dubois, K.A. Perrault, P.-H. Stefanuto, S. Koschinski, M. Edwards, L. McGregor, J.-F. Focant, Thermal desorption comprehensive two-dimensional gas chromatography coupled to variable-energy electron ionization time-of-flight mass spectrometry for monitoring subtle changes in volatile organic compound profiles of human blood, *J. Chromatogr. A.* (2017), <https://doi.org/10.1016/j.chroma.2017.04.026>.
- [33] P. Mochalski, J. King, M. Klieber, K. Unterkofler, H. Hinterhuber, M. Baumann, A. Amann, Blood and breath levels of selected volatile organic compounds in healthy volunteers, *Analyst* 138 (2013) 2134–2145.