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ORIGINAL ARTICLE

Effect of age and storage conditions on the volatile organic compound profile of blood

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Abstract Cadaver-detection dogs are used by the police to locate missing persons, victims of homicide, and human remains following mass disasters. Training is conducted using a variety of training aids including blood which can be hours, weeks or months old and stored under variable conditions. The aim of this study was to chemically profile human blood using solid-phase microextraction coupled with gas chromatography–mass spectrometry to determine how the volatile organic compound (VOC) profile changed over time and under variable storage conditions. The VOC profiles of fresh and degraded blood were analyzed as well as blood stored at room temperature, refrigerated, and frozen. Fresh and degraded blood samples produced distinctive VOC patterns with VOC profiles becoming more complex over time. Freezing the blood produced a complex VOC profile that was clearly discriminated from the VOC profile for blood stored at room temperature or in a refrigerator. This study highlights the importance of standardizing the age and storage conditions when using blood as a training aid to ensure cadaver-detection dogs are exposed to an accurate representation of the blood VOCs they may encounter at a scene.

Keywords Decomposition chemistry · VOC profile · Blood detection · Solid phase microextraction · Gas chromatography–mass spectrometry · Cadaver-detection dogs

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Introduction

Scent-detection canines are an integral tool used by many law enforcement agencies to assist in locating a range of target sources including drugs, explosives, currency, accelerants, and living and deceased individuals [1]. Canines used to locate deceased individuals or decomposed remains are typically referred to as human remains detection dogs (HRD dogs) or cadaver-detection dogs [1–3]. A need for this specialized group of scent-detection canines was recognized when police observed that search and rescue dogs had difficulty tracking an individual following death, indicating a clear behavioral distinction of the canines in response to different odor sources [1]. These observations have initiated multiple studies over the past decade to determine the chemical composition of decomposition odor and the key compounds that detection dogs utilize for scent recognition [1, 2, 4–9].

An odor profile comprises a range of volatile organic compounds (VOCs) in a gaseous state [6]. Canines are able to recognize VOCs from different odor profiles in order to track scents to their target sources [10]. Their sensitivity to odors can be attributed to their highly developed olfactory system and the large number of olfactory receptor cells within their nose, approximately 20–60 times greater than humans [11]. During the process of sniffing, VOCs bond to the odor receptors in the neurons, sending an electrical signal which is processed by the olfactory bulb of the brain [10]. Chemical properties such as molecule size, shape, stereochemistry, solubility, volatility, and polarity will determine the pattern of neuron firing, producing different electrical signals in the brain and creating a recognizable identifier for a particular odor [10].

Cadaver-detection dogs are used to locate missing persons, victims of crime, and victims of natural or man-made disasters [12]. Depending on the scene they are deployed

to, they may be tasked with searching and locating human cadavers or human remains such as tissue, blood, bone, and decomposition fluids [1]. Immediately following death, VOC production is minimal, but the VOC profile increases rapidly during the bloat and decay stages of decomposition, before declining throughout the skeletal stage [7]. The intensity and chemical profile of the odor is dynamic and constantly changing with time. In order to be effective, a cadaver-detection dog needs to be able to recognize a range of VOCs present throughout the various stages of decay [10].

One of the difficulties associated with training cadaver-detection dogs is sourcing suitable training aids. Cadaver-detection dogs are trained using a wide variety of materials ranging from natural scents such as flesh, blood, bone, teeth, soil or decomposition fluid to artificial scents such as putrescine, cadaverine, and commercial corpse scents [1, 6, 7, 10, 13]. Natural training aids are considered to be the most effective but their use and acquisition is often limited by legal and ethical restrictions. Few studies have investigated the odor profiles of individual human tissue types but it has been documented that there are VOC profile variations between muscle, adipose tissue, adipocere, bone, blood clots, and whole blood [5], highlighting potential issues when implementing cadaver-detection dog training materials.

Blood is a common training aid because it can be collected from a live and consenting donor which reduces the ethical issues associated with obtaining training materials from deceased persons. However the source, storage, and age of the blood samples can vary considerably between training units and the impact of these variables on the VOC profile and the response by the cadaver-detection dog is unknown. When presented with fresh and aged dried blood on carpet during training, cadaver-detection dogs have been observed to alert to aged samples but display difficulty in detecting fresh samples [14]. When the VOC odor profiles of the samples were further examined using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC–MS), the results demonstrated distinct VOC profiles for the fresh and aged dried blood, explaining the lack of response by the dogs to the fresh dried blood samples [14].

HS-SPME is particularly suited to analyzing blood VOCs [15, 16] because it does not require lengthy sample preparation and its non-contact nature reduces the hazards associated with analyzing human biological fluids. SPME uses a narrow fiber coated with a liquid polymer or a solid sorbent which absorbs/adsorbs the analytes proportional to their concentration in the sample. There are several different fiber coatings and combinations commercially available on the market, each with varying polarity, coating stability, maximum exposure temperature and analytical application and their use will depend on the technique and analytes of interest [17]. SPME has several advantages over other collection methods (e.g., sorbent tubes) including faster sampling, fewer labor requirements, small sample sizes, and no solvents which reduces the risk of contamination and is less destructive to the sample [18, 19]. SPME reduces background interference to improve reliability of compound identification and allows quantification of VOCs [20, 21].

The analysis of VOCs in blood using HS-SPME has been carried out extensively in the medical sciences to identify specific indicator compounds (e.g., biomarkers of lung cancer patients) or contaminants from the workplace or environment [8, 18, 21–25]. In the forensic sciences, only a limited number of studies have been conducted to profile blood VOCs [5, 8, 14]. These studies have demonstrated that the VOC profile of blood can vary significantly, which may result from the variations in an individual's genetics, microbiome, diet, living environment, and other external factors.

Through collaborations with police canine units in Australia, our research team has identified several anomalies in the age and storage of blood samples used as training aids for cadaver-detection dog training. It has previously been reported that cadaver-detection dogs failed to alert to fresh tissue types in the field due to training established on tissue that was hours, weeks or months old and stored under variable conditions [3, 14, 26, 27]. The purpose of this study was to chemically profile the headspace of blood samples collected from living donors to determine the variation in the VOC profile based on age of the sample and/or storage conditions. In order to accurately determine these variations, the current study collected samples from a single individual for each of the variables investigated as this minimized the number of variables which could not be accounted for.

Materials and method

Blood sample collection

Blood was acquired following institutional ethics committee approval and collected aseptically through venipuncture by qualified phlebotomists in Vacutainer® Tubes and Lavender Hemogard™ Closures (Becton, Dickinson and Company, NJ, USA). These Vacutainers® contained glass and spray-coated tripotassium ethylenediaminetetraacetic acid (K_3 EDTA). The effect of K_3EDTA on the blood VOC profile was tested and determined to have no significant effect above background levels. Blank Vacutainers® were also tested and shown to produce minimal background VOCs. The two donors for each trial were female, aged between 25 and 35 years, and were not taking any medication for health-related problems at the time of collection. Neither donor was

requested to change their normal daily routine and therefore applied soap and deodorant during their normal washing procedures on the day of sample collection. Neither donor was wearing perfume or any other odor detectable to the human nose.

Optimization of SPME extraction conditions

Three commercially available fiber types were assessed during optimization; 100 μm polydimethylsiloxane (PDMS) fused silica fiber, 65 μm PDMS/divinylbenzene (DVB) fused silica fiber, and a 50/30 μm DVB/carboxen (CAR)/PDMS StableFlex™ fiber (all 24-gauge). Fibers were thermally conditioned and confirmed blank prior to analysis of blood samples. During optimization, each fiber was exposed to 1.5 mL of blood in an airtight 20 mL glass SPME vial, sealed with a screw cap containing a polytetrafluoroethylene/silicone septum (Supelco, PA, USA). The volume of blood was chosen to optimize the number of replicates available for analysis from a single donor collection.

The PDMS fiber yielded fewer compounds due to its nonpolar phase and inability to absorb polar VOCs and was therefore excluded from further optimization. The bipolar PDMS/DVB and DVB/CAR/PDMS fibers were further tested at four different exposure times: 15, 30, 45, and 60 min. For both fiber types, the 15 min exposure time resulted in fewer compounds being detected than the longer exposure times. Although the number of compounds was comparable, the peak shape and resolution was superior for the 45 min exposure when compared to the 30 and 60 min exposure.

Three temperatures were also investigated for the volatilization of the VOCs from the blood; 25, 40, and 60 °C. Exposure at 25 °C was ineffective and resulted in very few compounds being detected. Heating the blood samples to 60 °C during exposure adversely affected the VOC profile as many compounds degraded at this temperature. The optimum exposure temperature was determined to be 40 °C. The final optimized SPME parameters used for all sample analyses involved a 65 μm PDMS/DVB fiber exposed to 1.5 mL of blood for 45 min at 40 °C.

GC–MS analysis

This study involved a nontarget approach in order to chemically profile the spectrum of blood VOCs present in samples that were aged and stored under variable conditions. It was not possible to analyze chemical standards for every VOC that could be identified. Instead a review of the literature was conducted to identify common VOCs reported in blood profiling studies and cadaver-dog training studies [5, 8, 15, 18, 21, 28]. A mixed standard incorporating reported VOCs was prepared for analysis and included 2-methylpentane, heptane, undecane, 1-pentanol, 2-ethyl-1-hexanol, 1-hexanol, hexanal, heptanal, octanal, 2-pentanone, 2-butanone, 2-nonanone, *p*-xylene, *o*-xylene, toluene, ethylbenzene, benzene, styrene, tetrachloroethylene, dimethyl disulfide and dimethyl sulfide. The mixed standard represented the range of compound classes anticipated in the blood VOC profile. The retention time and mass spectra of available compounds was compared between standards and blood samples to improve peak identification.

A fiber blank was analyzed at the beginning of each sample analysis and after every third sample to confirm the absence of cross-contamination. A control sample containing 1.5 mL of saline solution (0.9 % sodium chloride analytical reagent) was also analyzed on each day of analysis. Prior to sample collection, 10 μL of 30 ppm bromobenzene in methanol (HPLC grade) was added to each control and sample as an internal standard.

Sample analysis was carried out using a 7890A Gas Chromatograph coupled to a 5975C Mass Selective Detector (Agilent Technologies, Palo Alto, CA, USA). Compounds were separated using a HP-5MS column (30 m × 0.250 mm ID × 0.25 μm film, 5 % phenyl/methylpolysiloxane) (J & W Scientific, CA, USA). Helium was used as the carrier gas at a flow rate of 1.5 mL/min and all analyses were performed in splitless mode. Compounds were desorbed in the GC inlet at 200 °C for 5 min. The GC oven started at an initial temperature of 35 °C held for 1 min, then was ramped to 80 °C at 3 °C/min, followed by a second ramp to 180 °C at 10 °C/min, and a final ramp to 260 °C at 40 °C/min. The total analysis time was 23.5 min. The MS was operated in full electron ionization (EI) scan mode with a mass range of 40–450 m/z.

Data analysis was performed using Agilent Chemstation and compounds were identified using the National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST 05). A minimum match threshold of 70 was selected. Compounds were removed during preprocessing if they were below this threshold, present at comparable levels in control (saline) samples, or if they resulted from fiber sorbent or column bleed. Compounds identified from potential environmental contamination were noted and documented separately in Table 1. These were compounds that have been previously reported in the literature as resulting from environmental sources and may include exogenous sources entering the blood, or those that are naturally present in the laboratory environment (including fume hood, refrigerator, and freezer). Internal standard normalization was performed on the peak area of target compounds; those compounds found exclusively in the experimental blood samples, or in higher abundance than the control and fiber blanks, have been compiled in Table 1.

Table 1 The major VOCs detected in all blood samples and classified by chemical classes

	Age of blood		Storage condition			References previously citing VOC in blood[a]
	Fresh (0–48 h)	Degraded (1–6 weeks)	RT (1–4 weeks)	Fridge (1–5 weeks)	Freezer (1–5 weeks)	
Aromatics						
Toluene				x	x	[5, 8, 15, 18, 21, 28, 39]
Ethylbenzene				x	x	[18, 21, 28, 39]
Xylene				x	x	[18, 28]
Styrene				x	x	[18, 21, 28, 39]
Benzene, (1-methylethyl)-				x		NR
Benzene, 1,2,3-trimethyl-				x		NR
Furan, 2-pentyl-		x	x		x	NR
Esters						
Butanoic acid, methyl ester			x			
Ethers						
Propane, 1,1-dimethoxy-2-methyl-			x	x	x	NR
2,2-Dimethoxybutane					x	NR
Aldehydes						
Hexanal		x				[5, 15, 16, 18, 28]
Heptanal		x			x	[16, 18, 28]
Octanal					x	[16, 28]
Decanal					x	[16]
Ketones						
3-Hexanone			x	x	x	NR
2-Heptanone	x	x	x	x	x	[15]
2-Heptanone, 6-methyl-		x			x	NR
4-Heptanone	x	x	x		x	NR
3-Octanone		x	x	x		NR
2-Nonanone		x	x			NR
2-Undecanone		x	x			NR
2,5-Octanedione		x	x	x	x	[18, 28]
5-Hepten-2-one, 6-methyl-		x				NR
Alcohols						
1-Pentanol		x			x	[15]
1-Hexanol		x			x	[5]
1-Hexanol, 2-ethyl-		x	x	x		[5]
1-Octen-3-ol	x	x	x	x	x	[5, 15, 28]
2-Octen-1-ol	x					[28]
Hydrocarbons						
Heptane					x	[28]
Octane					x	NR
Decane					x	NR
Tridecane					x	[16]
Dodecane		x		x	x	[16]
Nonadecane					x	NR
2-Octene					x	[28]
Dodecene					x	[15]
Environmental compounds						
Diphenyl ether	x	x	x	x	x	NR
Eucalyptol	x	x	x	x	x	NR

Table 1 continued

	Age of blood		Storage condition			References previously citing VOC in blood[a]
	Fresh (0–48 h)	Degraded (1–6 weeks)	RT (1–4 weeks)	Fridge (1–5 weeks)	Freezer (1–5 weeks)	
Limonene	x	x	x	x	x	NR
Menthol		x	x	x	x	NR
Menthone			x	x	x	NR
Carvone			x	x	x	NR
Aromadendrene					x	NR
4-Cyanocyclohexene			x	x		NR
Cycloisolongifolene, 8,9-dehydro-	x	x	x	x	x	NR
1,5,9-Cyclododecatriene, 1,5,9-trimethyl-	x	x	x	x	x	NR

[a] *NR* not reported in literature as a VOC detected in blood

Principal component analysis (PCA) was used to visualize the structure of the data for the blood variables of interest (blood age and storage condition). Normalized peak areas for each compound was input into The Unscrambler X version 10.3 (CAMO Software). Data groupings can be examined on score plots. Loading plots indicate the key compounds responsible for affecting scores and causing noticeable data groupings. This made it possible to discern whether the blood VOC profile was changing over time or with storage condition and to determine the key compounds that affect this change based on the visual representation of the points.

Trial 1: Aging blood samples

The first trial involved chemically profiling blood that had been stored at room temperature over a period of 6 weeks to investigate the effect of age on the VOC profile. Anecdotal information from local police canine units had indicated that blood collected from a live donor is often used as a training aid for up to 4–6 weeks after collection.

Following collection from Donor 1, the blood was subsampled into sterile 20 mL sealed SPME vials for subsequent aging and analysis of replicates. The term "fresh blood" was used to describe blood that was sampled and analyzed within 48 h of collection. The term "degraded blood" was used to describe blood that was sampled and analyzed after 48 h of collection and storage. Fresh blood was analyzed immediately after collection (0 h) and after 24 and 48 h storage at room temperature. Thereafter, degraded blood was analyzed weekly for a further 6 weeks. Each sample analysis involved three replicates, one fiber blank, and one control sample.

Trial 2: Storage conditions

The second trial involved chemically profiling blood that had been stored under different conditions. Anecdotal information from local police canine units indicated that blood is typically stored as a training aid at room temperature, but in some instances may also be stored in a refrigerator or freezer depending on the storage facilities available. Samples stored in a refrigerator or freezer were typically only held for 4–5 weeks due to separation of the blood cells from the serum and concerns about their value as training aids thereafter.

Following collection from Donor 2, the blood was subsampled into separate 20 mL sealed SPME vials for subsequent storage of replicates at room temperature, in a refrigerator, and in a freezer. Samples stored at room temperature and in the refrigerator were analyzed weekly for 4–5 weeks. Samples stored in the freezer were analyzed biweekly for the same period of time. To ensure all the vials were analyzed in a similar state, the vials stored in the refrigerator or freezer were allowed to return to room temperature prior to analysis. This also mimicked the condition of the training aids when presented to cadaver-detection dogs during training. Each sample analysis involved three replicates, one fiber blank, and one control sample.

Results

Trial 1: Aging blood samples

The major VOCs observed in all blood sample variables were classified by chemical classes as outlined in Table 1. VOCs detected in the first 48 h of collection were classified

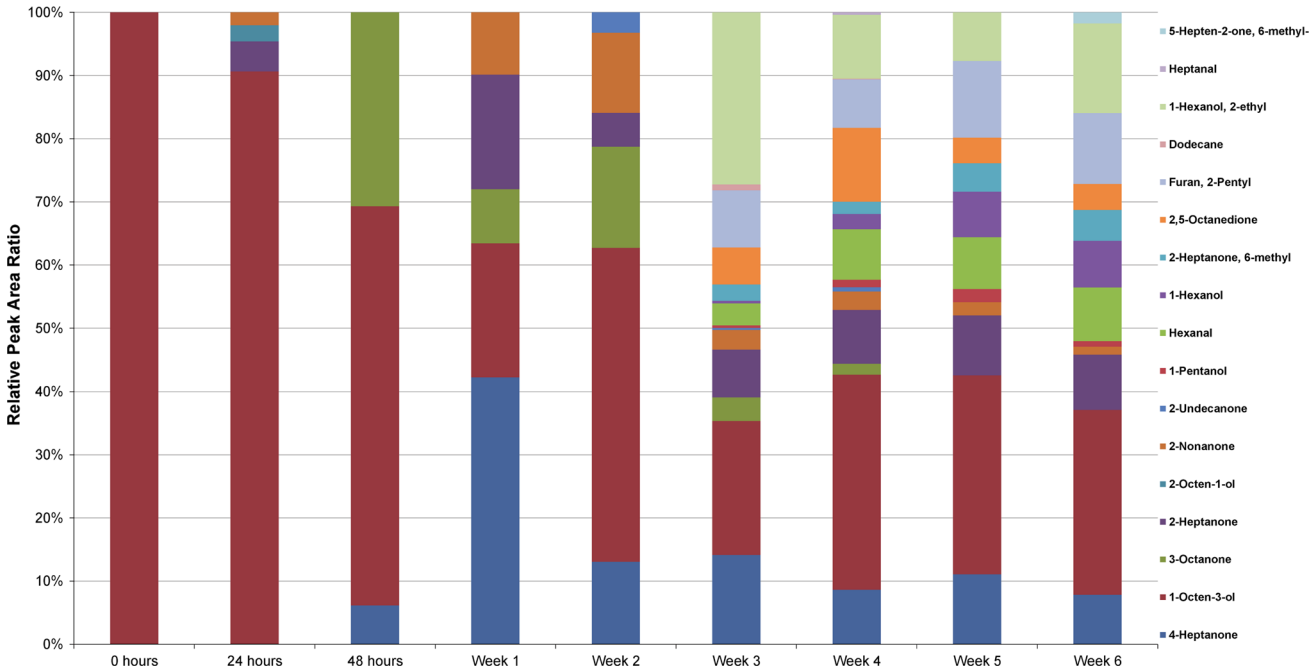


Fig. 1 VOC profile of fresh and degraded blood demonstrating the number and relative peak area ratio of compounds detected over time (excluding those compounds originating from potential environmental sources)

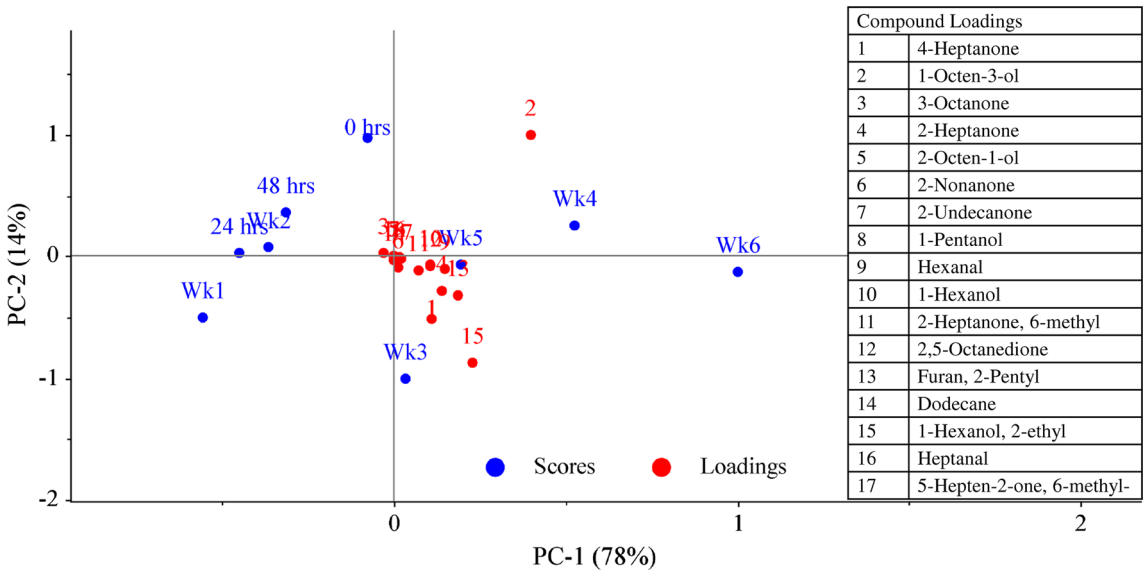
under "fresh blood" and VOCs detected after 48 h of collection were classified under "degraded blood". Compounds believed to be exogenous and resulting from environmental sources are listed separately. References where compounds have been previously cited were also reported.

The VOC profile of fresh and aged blood showed distinct differences in chemical compound patterns and concentration with degraded blood demonstrating an increased complexity over time. Figure 1 displays the number and relative ratio of compounds detected for each sampling period, excluding those compounds believed to originate from environmental sources. Fresh blood consisted predominantly of alcohols and ketones with 1-octen-3-ol representing the only compound detected immediately after blood collection, and the largest component during the first 48 h. Degraded blood demonstrated a higher proportion of ketones, alcohols and aldehydes although 1-octen-3-ol remained a dominant component in the blood VOC profile throughout the 6 week study. The VOC profile of the fresh blood changed gradually from 48 h to 2 weeks after collection with 4-heptanone, 3-octanone, 2-heptanone, 2-nonanone, and 2-undecanone appearing. Thereafter, an increased number of ketones, alcohols, and aldehydes were detected in varying ratios until the end of the study. In particular, 2-pentylfuran and 2-ethyl-1-hexanol became prevalent VOCs from week 3 onwards.

PCA plots demonstrating the relationships between the aged blood samples (scores) and the VOCs detected for these samples (loadings) are shown in Fig. 2. These plots include all VOCs listed in Table 1, with the exception of the compounds believed to originate from environmental sources. Due to the reduced complexity of the VOC profile, those samples classed as fresh blood (24 and 48 h) clustered closely, with the exception of blood analyzed immediately after collection (0 h). 1-octen-3-ol was the only VOC detected in this sample and was also discriminatory in many of the degraded blood samples. The blood VOC profiles for week 1 and 2 were clearly separated from weeks 3 to 6 demonstrating the variance as the blood samples aged. VOCs largely responsible for distinguishing the degraded blood samples were: 1-octen-3-ol, 2-heptanone, 4-heptanone, 2-ethyl-1-hexanol, and 2-pentylfuran. These VOCs demonstrated high discriminatory power as the age of the blood increased.

Trial 2: Storage conditions

Table 1 and Fig. 3 highlight the variation in the VOC profiles detected across the three storage conditions with time. The room temperature samples contained a higher number of VOCs, even on the day of collection, than the equivalent samples in the aging study. In addition to 1-octen-3-ol which was a major VOC in all of the room temperature samples, the blood samples analyzed immediately after collection also contained 2-heptanone, 3-octanone, and butanoic acid methyl ester. The complexity of the VOC profile gradually increased over the next 4 weeks with the addition of 2-nonanone, 2,5-octanedione, 2-undecanone, 2-ethyl-1-



Compound Loadings	
1	4-Heptanone
2	1-Octen-3-ol
3	3-Octanone
4	2-Heptanone
5	2-Octen-1-ol
6	2-Nonanone
7	2-Undecanone
8	1-Pentanol
9	Hexanal
10	1-Hexanol
11	2-Heptanone, 6-methyl
12	2,5-Octanedione
13	Furan, 2-Pentyl
14	Dodecane
15	1-Hexanol, 2-ethyl
16	Heptanal
17	5-Hepten-2-one, 6-methyl-

Fig. 2 PCA scores and loadings bi-plot for VOCs detected in fresh (0, 24, 48 h) and degraded (week 1–6) blood based on their average relative peak area

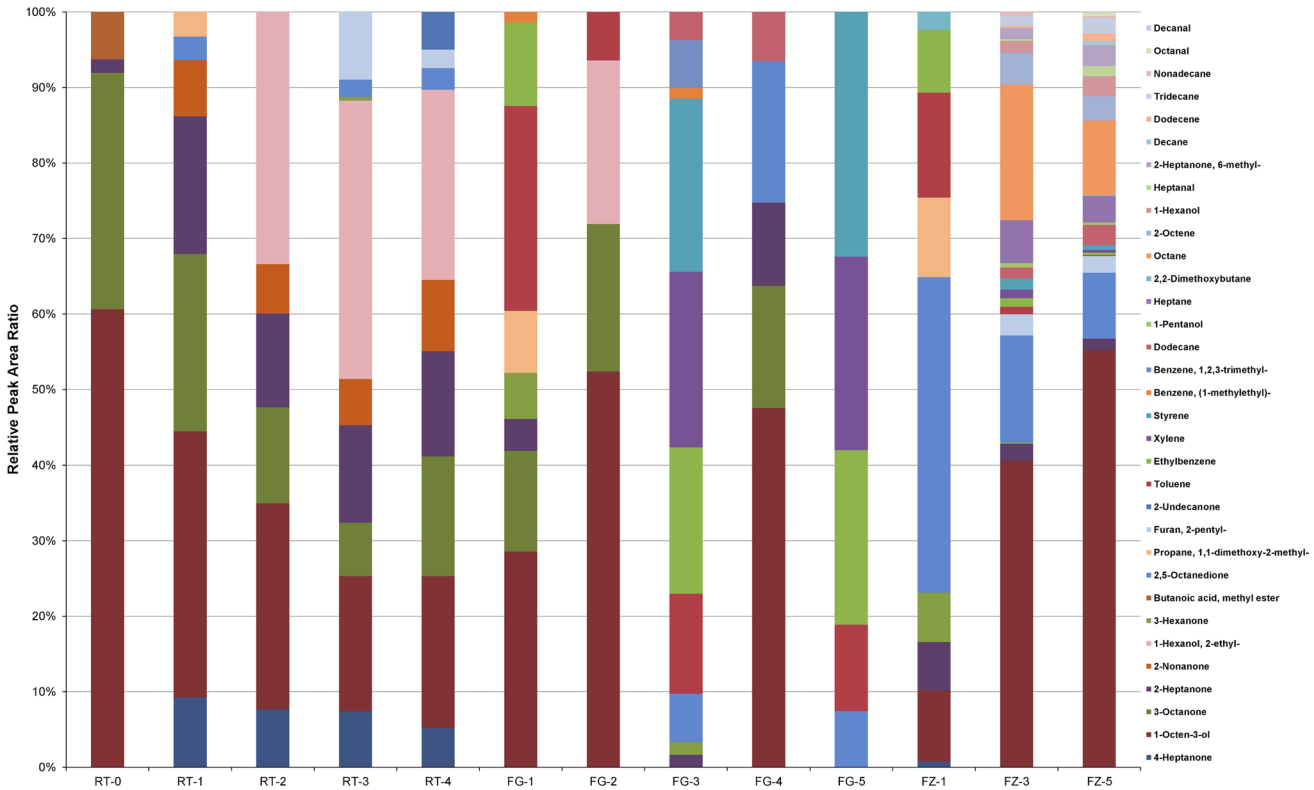


Fig. 3 VOC profile of blood stored at room temperature (RT), in the refrigerator (FG), and in the freezer (FZ), demonstrating the number and relative peak area ratio of compounds detected from 0 to 5 weeks after collection (excluding those compounds originating from potential environmental sources)

hexanol, 2-pentylfuran, and 1,1-dimethoxy-2-methylpropane but when compared to the refrigerator and freezer samples, demonstrated a relatively simple VOC profile.

The blood samples stored in the refrigerator demonstrated highly variable VOC profiles with few VOCs consistently detected over time. Notably, 4-heptanone was not

detected in any of the refrigerated samples suggesting a lack of formation at cooler temperatures. 1-octen-3-ol and 3-octanone were prevalent VOCs in the samples refrigerated for 1, 2, and 4 weeks but were not detected in the samples refrigerated for 3 and 5 weeks. The absence of these compounds after 3 and 5 weeks storage in the refrigerator cannot be confirmed, particularly since they were detected in the 4 week storage samples. Their absence may be the result of masking by other more dominant VOCs. The samples analyzed after 3 and 5 weeks storage in the refrigerator demonstrated very similar VOC profiles to each other, dominated by aromatic compounds including toluene, ethylbenzene, xylene, and styrene (commonly referred to as BTEXS when benzene is also detected). Although BTEXS are typically associated with environmental sources, these compounds were included in Fig. 3 as they have been previously reported in blood VOC profile studies (see Table 1).

The samples stored in the freezer also demonstrated variable VOC profiles. 1-octen-3-ol, 2-heptanone, and 2,5-octanedione were detected in the blood samples stored for 1, 3, and 5 weeks. Toluene, ethylbenzene, xylene, and styrene were also consistently detected in samples stored for 3 and 5 weeks. When compared to the room temperature and refrigerator storage conditions, the freezer storage conditions produced highly complex VOC profiles, specifically after 3 and 5 weeks storage. The profiles were dominated by aromatics, ketones, hydrocarbons (predominantly alkanes and alkenes), aldehydes, and alcohols. Hydrocarbons were detected in much higher numbers in the freezer samples with dodecane being the only hydrocarbon detected in the room temperature and refrigerated samples.

A PCA plot demonstrating the relationship between the storage conditions (scores) and the VOCs detected for these samples (loadings) is shown in Fig. 4. The plot includes all VOCs listed in Table 1, with the exception of the compounds believed to originate from environmental sources. All room temperature and refrigerated samples clustered closely suggesting minimal variation in their VOC profile. The blood samples stored in a freezer for 1 week were also similar in profile however the VOC profile became increasingly more complex after the samples were stored for 3 and 5 weeks, respectively. This discrimination was largely due to the presence of C8 compounds; octane, 2,5-octanedione, and 1-octen-3-ol, the latter being detected in most samples.

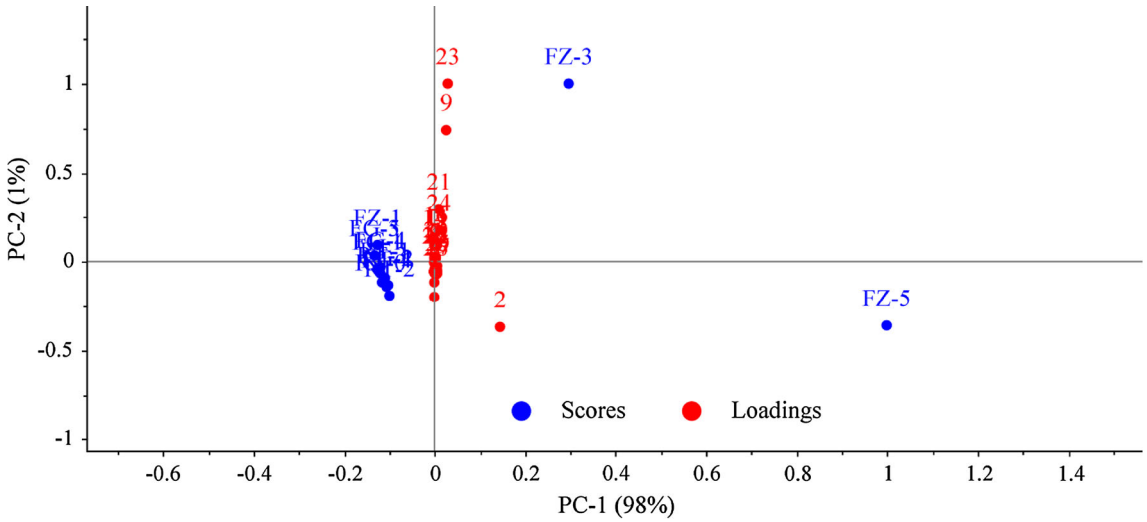
In order to better visualize any discrimination between the room temperature and refrigerated samples, the freezer samples were removed and PCA plots regenerated as shown in Fig. 5. The new plots demonstrated discrimination of the points with clustering for the aged room temperature samples (RT-2, RT-3, RT-4). 1-octen-3-ol, 2-heptanone, 3-octanone, and 2-ethyl-1-hexanol had the highest discriminatory power for these samples, and were comparable to the discriminatory VOCs observed for the aging trial at the same time. Notably, the samples stored in the refrigerator for 3 and 5 weeks are clearly discriminated from the other refrigerated samples, largely due to the presence of toluene, ethylbenzene, xylene, and styrene. These plots correlate with the results in Fig. 3 suggesting that the presence of these VOCs is anomalous and not consistent with the typical VOC profile for blood samples stored in a refrigerator.

Discussion

The first trial identified differences in blood VOC profile over time and utilized storage conditions most commonly reported by police canine units in Australia (i.e., stored at constant room temperature). The second trial repeated the first study at room temperature and identified differences in the blood VOC profile based on alternative storage conditions reported by police canine units (i.e., refrigerated and frozen). No single VOC was detected in all samples, although 1-octen-3-ol and 2-heptanone are reported in all columns of Table 1. The table represents a summary of the compounds detected in each trial but analysis of Figs. 1 and 3 is required to determine their presence in all samples. The most prevalent compound detected was 1-octen-3-ol which was present in all samples analyzed except the blood samples stored in the refrigerator for 3 and 5 weeks. However, given the anomalous VOC profiles for these particular weeks, the absence of 1-octen-3-ol in these samples cannot be confirmed as the compound peak may have been masked by other dominant compounds which appeared only in these profiles. 2-heptanone was also prevalent in most samples but was not detected at 0 h or 48 h of the aging trial (although it was detected at 0 h of the storage condition trial at room temperature), nor was it detected in the samples refrigerated for 2 and 5 weeks. The only VOC to be detected in all blood samples analyzed was diphenyl ether, which is a common ingredient in soaps, detergents, lotions, and perfume [29] and was therefore deemed to result from an exogenous source.

Trial 1: Aging blood samples

The high abundance of 1-octen-3-ol in the samples analyzed immediately after collection could be attributed to the oxidative breakdown of fatty acids in the blood, specifically linoleic acid. 1-octen-3-ol has been reported as a VOC in blood in several other studies [5, 15, 28] and is also present in human breath, saliva, sweat and the headspace of muscle tissue [5]. It can be produced by plants and fungi and has been associated with mold emissions in fungal growths [30]. This may account for the ongoing presence of 1-octen-3-ol even after oxidative breakdown occurred as



Compound Loadings		17	Benzene, (1-methylethyl)-
1	4-Heptanone	18	Benzene, 1,2,3-trimethyl-
2	1-Octen-3-ol	19	Dodecane
3	3-Octanone	20	1-Pentanol
4	2-Heptanone	21	Heptane
5	2-Nonanone	22	2,2-Dimethoxybutane
6	1-Hexanol, 2-ethyl-	23	Octane
7	3-Hexanone	24	2-Octene
8	Butanoic acid, methyl ester	25	1-Hexanol
9	2,5-Octanedione	26	Heptanal
10	Propane, 1,1-dimethoxy-2-methyl-	27	2-Heptanone, 6-methyl-
11	Furan, 2-pentyl-	28	Decane
12	2-Undecanone	29	Dodecene
13	Toluene	30	Tridecane
14	Ethylbenzene	31	Nonadecane
15	Xylene	32	Octanal
16	Styrene	33	Decanal

Fig. 4 PCA scores and loadings bi-plot for blood stored at room temperature (RT), in the refrigerator (FG), and in the freezer (FZ) based on the average relative peak area of compounds detected from 0 to 5 weeks after collection

fungal growths were observed in the degraded blood samples later in the trial.

The fresh blood samples produced the simplest VOC profile which may be attributed to low bacterial and fungal activity as a result of the aseptic techniques used for sample collection into the Vacutainers® and the minimal storage time in the sealed vials prior to collection of the headspace. Only four VOCs believed to be endogenous blood compounds (and not resulting from environmental sources) were detected, three of which have been previously reported [5, 15, 28]. 4-heptanone has not been previously reported in the cited literature for blood but is a volatile ketone found in the urine of normal individuals [28]. Its absence during the first 24 h suggests its formation over time resulting from enzymatic or bacterial activity. Anecdotal evidence suggesting a failure to alert on fresh blood by cadaver-detection dogs in the field may be a result of the simple VOC profile exhibited during this time. The VOC profile of fresh blood could be difficult to distinguish from environmental odors, as an equal number of environmental VOCs were detected in the fresh blood, which may reduce the specificity of the canine olfactory processes.

Degraded blood displayed greater VOC complexity than fresh blood with a wider range of chemical classes detected. This complexity was predominantly observed after the samples aged for 3 weeks at room temperature. The increase in VOC complexity could be attributed to bacterial activity and/or enzymatic degradation of proteins in the blood [5]. In addition to the VOCs detected in fresh blood samples, those compounds which clearly discriminated the degraded blood included 2-ethyl-1-hexanol and 2-pentylfuran. Hoffman et al. [5] reported the presence of 2-ethyl-1-hexanol in blood although it was only detected in whole blood samples and not in the blood clot or placenta samples

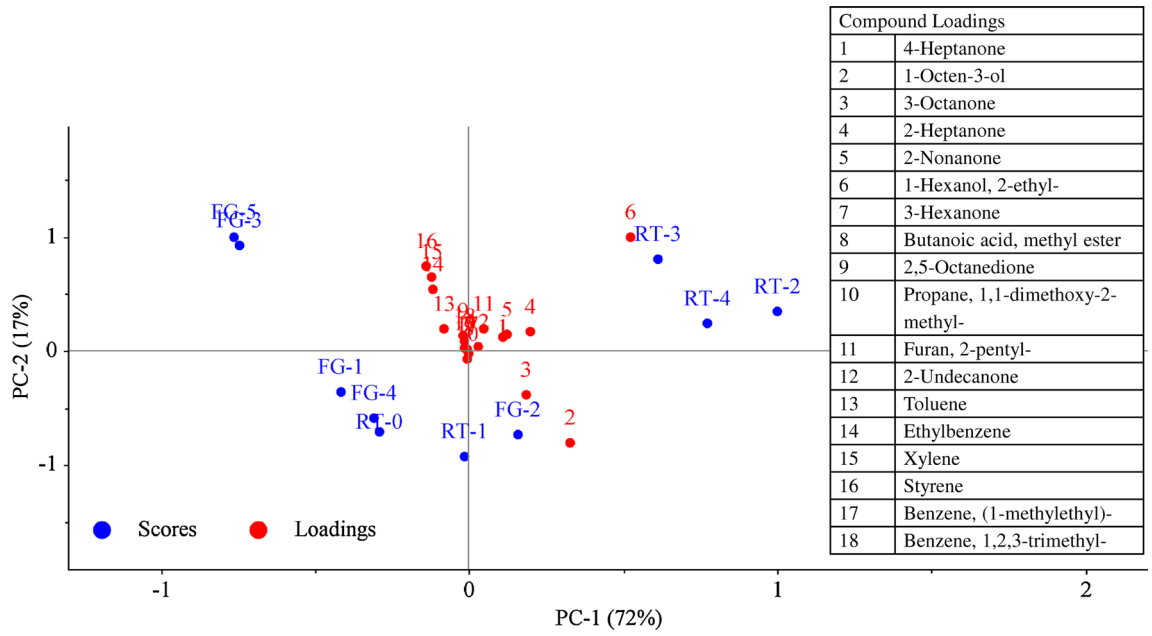


Fig. 5 PCA scores and loadings bi-plot for blood stored at room temperature (RT) and in the refrigerator (FG) based on the average relative peak area of compounds detected from 0 to 5 weeks after collection

also analyzed. Its presence in blood is not surprising given its large scale production for use in fragrances, flavors, and solvents. It is also used as a precursor for plastics and has been reported as a common additive in plasticized explosives, representing one of the dominant VOCs available for explosive-detection canines [31]. 2-pentylfuran was also reported by Hoffman et al. [5] in all soft and hard tissues analyzed but it was not detected in blood. Alkyl furans are often produced by fungi [28] which may explain the presence of 2-pentylfuran from week 3 onwards in all of the blood samples maintained at room temperature, as fungal growth was observed in these vials.

Aldehyde compounds, specifically hexanal and heptanal, were only identified in the degraded blood samples. Both VOCs have been reported as potential biomarkers of lung cancer when detected in high concentrations in living patients [18] although hexanal is commonly detected in a range of biological fluids [15]. Hexanal was identified in the VOC analysis of blood used for training cadaver-detection dogs [5] however the age of the training aids used in the study is unknown. Their presence may reflect storage of the sample prior to use as aldehydes only appeared following 3 weeks storage at room temperature in the current study.

It is currently unknown whether cadaver-detection dogs utilize individual VOCs or a combination of VOCs when alerting to the presence of blood or human remains. While fresh blood exhibited a very simple VOC profile, it may contain a key compound capable of eliciting a response by cadaver-detection dogs. Alternatively, a more complex VOC profile might result in saturated bonding of compounds to canine receptors, exhibiting a pattern more commonly associated with decomposition. This could explain why degraded blood has been more readily located than fresh blood (based on anecdotal evidence). Until the VOCs of interest are identified conclusively, training of cadaver-detection dogs using both fresh and degraded blood will ensure the canines are exposed to the broad spectrum of VOCs produced by blood stored for up to 6 weeks.

Trial 2: Storage conditions

The VOC profiles of the blood samples stored at room temperature demonstrated an increased complexity with time, particularly during weeks 2–4. The PCA plots produced a clustering of these weeks demonstrating similarities in the ratio of VOCs detected. As the samples aged, their VOC profiles also resembled those observed during trial 1.

The VOC profiles of the blood samples stored in a refrigerator were more variable. The VOC profiles for the samples stored for 1, 2, and 4 weeks were similar to each other and to the room temperature samples analyzed after 0 and 1 week storage. This finding suggests that the cooler temperatures in the refrigerator were sufficient for reducing biological degradation due to the suppression of enzymatic, bacterial and fungal activity within the blood samples. It is generally acknowledged that the blood stream in a healthy individual is sterile [32] and it is therefore unlikely that

bacterial degradation would have resulted, particularly at the lower temperatures.

The VOC profiles for the samples stored in the refrigerator for 3 and 5 weeks were determined to be anomalous due to the presence of toluene, ethylbenzene, xylene and styrene. These VOCs can be found in a range of solvents [33] and are also common in indoor environments and food products. Chambers et al. [34] found that positive contamination of BTEXS can result from the Vacutainers® into which blood is collected and stored. Based on their studies, the BTEXS residues in the butyl rubber stoppers of Vacutainers® are the most persistent at low levels [35]. They also demonstrated that these VOCs can concentrate in refrigerated air, demonstrating higher levels of BTEXS for samples stored in a refrigerator that were not hermetically-sealed. The anomalous appearance of toluene and ethylbenzene in weeks 1, 3, and 5, along with the appearance of xylene and styrene in weeks 3 and 5 of the refrigerated samples suggests a degree of contamination in the current study, either from the Vacutainers® or the air within the refrigerator. Since these compounds did not appear in all samples or above background levels in the control (saline) samples, contamination was limited and the VOC profiles from the blood stored in a refrigerator for 1, 2, and 4 weeks, respectively, are considered to be representative.

The blood samples stored in the freezer were analyzed less frequently than the room temperature and refrigerated samples because it was originally hypothesized that the frozen samples would degrade at a slower rate and demonstrate minimal variation in VOC profile over time. However, the results indicate that freezing the blood samples increased the rate of degradation, potentially due to the lysing of blood cells following freezing [36]. Typically centrifugation and separation of blood fractions is recommended prior to storage in a freezer, and plasma and serum particularly should be stored at −80 °C or lower [37]. However, such storage conditions are not representative of the facilities available at police dog units and as a result, storage of whole blood samples at −20 °C was investigated in this study.

Toluene, ethylbenzene, xylene, and styrene were detected in the samples stored in the freezer suggesting a degree of contamination similar to the refrigerated samples. The complexity of the VOC profile after 3 and 5 weeks storage in the freezer predominantly resulted from the high proportion of straight-chain alkanes, specifically heptane, octane, decane, dodecane, tridecane, and nonadecane. The analysis of n-alkanes has been carried out to determine picogram levels in blood resulting from non-occupational exposure to common sources such as petroleum-based fuel [38]. However, the absence of these alkanes in the blood samples analyzed immediately after collection from Donor 2 suggests that the presence of alkanes in the frozen samples is more likely a result of cell lysis and degradation, rather than exposure to external sources.

The storage of blood samples under different conditions demonstrated a variability in the VOC profile detected which could not be attributed solely to the aging of the sample with time. The samples stored in the freezer yielded a much more complex VOC profile which was inconsistent with samples aged for the same period of time at room temperature and in the refrigerator. The results suggest that storage of blood samples at room temperature or in a refrigerator provide more comparable VOC profiles for training cadaver-detection dogs while storing blood samples in a freezer may produce VOCs that are not truly reflective of a blood odor that would be encountered at a crime scene.

Conclusion

Anecdotal evidence has suggested variable responses by cadaver-detection dogs to blood training aids that have been stored for different periods and under different conditions. This study involved chemically profiling blood samples aged for up to 6 weeks and stored under different conditions, i.e., room temperature, in a refrigerator, and in a freezer, to determine whether the VOC profiles varied with time. The VOC profile of blood stored at room temperature was initially relatively simple but became more complex as it aged. This finding may assist in explaining why cadaver-detection dogs have reportedly found it difficult to alert to fresh blood compared to aged or degraded blood during training. Until the key VOCs that elicit an alert are identified, the use of both fresh and degraded blood as training aids is advised to ensure cadaver-detection dogs are exposed to the broad spectrum of blood VOCs they may encounter at a crime scene. Storage of blood samples at room temperature and in a refrigerator did not impact the VOC profile to the same extent as storing blood samples in a freezer. Based on the findings of this study, storage in a freezer is not recommended as it may negatively impact the VOCs available to cadaver-detection dogs during training. Storage at room temperature best mimics the conditions that cadaver-detection dogs will be exposed to, however storage in a refrigerator is occasionally necessary due to OH&S requirements and does not appear to significantly impact the VOC profile, assuming it remains tightly sealed in an appropriate container during storage.

Key Points

1. Solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC–MS) were used to analyze the volatile organic compounds (VOC) in blood.

2. Blood samples that had been aged and stored at room temperature, in a refrigerator, and in a freezer, were analyzed to identify differences in the VOC profiles.
3. Fresh and degraded blood demonstrated different VOC profiles.
4. Freezing the blood samples produced a VOC profile that was clearly distinct from samples stored at room temperature and in the refrigerator.
5. Variation in age and storage of blood samples can impact the VOC profile available to cadaver-detection dogs during training.

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