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TECHNICAL NOTE

GENERAL

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Decomposing Human Blood: Canine Detection Odor Signature and Volatile Organic Compounds*

ABSTRACT: The admissibility of human "odor mortis" discrimination in courts depends on the lack of comprehension of volatile organic compounds (VOCs) during the human decay process and of the lack in standardized procedures in training cadaver dogs. Blood was collected from four young people who died from traffic accidents and analyzed using HS-SPME/GC-MS at different decompositional stages. Two dogs, professionally trained, were tested to exactly locate blood samples, for each time point of the experiment. We found a long list of VOCs which varied from fresh to decomposed blood samples, showing differences in specific compounds. Dog performance showed a positive predictive value between 98.96% and 100% for *DOG A*, and between 99.47% and 100% for *DOG B*. Our findings demonstrated that decomposing human blood is a good source of VOCs and a good target for canine training.

KEYWORDS: forensic sciences, human scent, human blood, decompositional process, volatile organic compounds, canine detection

Canines (*Canis familiaris*) may represent a reliable source in forensic investigations because of the high sensitivity and selectivity of their olfactory system (1). If we consider that nearly one-eighth of their brain seems to be committed to this sense, a well-trained dog would be capable to identify a specific odor (2). In details, cadaver dogs may be trained to detect human remains or fluids.

However, the lack of standardized procedures in their training questioned the admissibility of human "odor mortis" discrimination in courts (1,3–12). Moreover, the ability to target transient odor compounds by these biological detectors is not fully understood. After death, indeed, human tissues are degraded by reactive oxygen species, autolysis, microorganisms, or scavengers, thus producing VOCs (13–25). These substances are intermediate decaying products of proteins, carbohydrates, nucleic acids, and lipids. Many studies investigated the VOCs released by decomposing human bodies and succeeded in identifying a large number of these compounds as human decomposition markers (2,13–15,17,18,25).

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VOCs are released into the environment, thanks to their physicochemical properties, such as low molecular weight, high vapor pressure, and low boiling point (16,24,26,27). Several different factors may influence the decompositional human process: temperature, humidity, barometric pressure, burial depth, air currents, soil composition, and moisture, which strongly affects VOCs profile (28). Other influencing factors are the presence or absence of clothing or wrappings, the percentage of body fat, diseases and the use of chemicals or drugs, insect activity, vegetation, and location (indoors vs. outdoors) (28).

The development of protocols for canine detection will allow odors to be accepted as forensic evidence. In order to contribute to this aim, this paper follows the ongoing research, directed toward the improvement and standardization of canine olfactive performance in detecting human cadaveric blood traces (29).

Previous research showed interesting results in the identification of human cadaveric blood, demonstrating that, after specific training, dogs can identify few and latent traces of blood with high precision in discriminating the human cadaveric blood among confounding, nontarget odors (29). In this phase of our research project, we aimed both to evaluate the performance of dogs, trained through a rigorously controlled design, and to identify the volatile organic compounds (VOCs) released by the same blood samples.

Material and Methods

Sample Selection

For our experimental study, blood was selected as VOCs source because of its presence both in all human tissues and often in a crime scene. Therefore, as outlined by Lorenzo et al.

(25), decaying human blood contains a wide range of the by-products of decomposition and putrefaction, so that it can be intended as the most authentic source.

We collected blood samples from deceased subjects from traumatic causes. Since the human odor may be influenced by several pathological conditions and metabolic pathways, we selected only samples from nondiseased subjects. Cases with positive toxicological screening were also excluded. Based on these criteria, we identified four young people who died from traffic accidents (one 22-year-old black man, one 22-year-old white man, one 24-year-old black woman, and one 20-year-old white woman). These blood samples were collected within 4–5 h from death from the internal jugular vein (nontraumatized areas) into vacutainer containing sodium fluoride and potassium oxalate using the same protocol at the same location and by the same team. Blood collection tubes were pretreated to remove VOC contaminants as described by Cardinali et al. (30).

Sample Preparation

The collected samples were then transferred to our laboratory on dry ice and stored at -20°C. The samples were then equilibrated at room temperature (22°C, 45% relative humidity). The maximum amount of time that the samples were maintained at room temperature was 24 h. For analysis, 0.5-ml of each blood sample was transferred by gastight syringe from the blood collection tube in two 10-mL glass vials with phenolic cap and PTFE/Silicone sept: one for GS-MS analysis and one for dog training procedure. Before this, the vials were cleaned with acetone and heated for 24 h at 200°C to remove any eventual contamination inside. The analytical cleanliness of the vials was determined by GC-MS prior to sampling. VOCs headspace (HS) was extracted by solid phase microextraction (SPME) using polydimethylsiloxane/divinyl-benzene (PDMS/DVB) 65 μm fibers and analyzed by GC-MS. GC-MS analyses were carried out on a Finnigan Trace DSQ GC-MS (Thermo Fisher Scientific, Austin, Texas). The GC-MS system was equipped with the DB5-MS capillary column, 30 m in length, 0.25 mm ID, and 0.25 μm film thickness. Helium carrier gas was set to 1.5 mL/min flow rate and GC oven was held at an initial temperature of 35°C for 1 min, ramped to 80°C at 3°C/min, then to 120°C at 10°C/min and finally to 260°C at 40°C/min. This qualitative method was based on a previously reported GC-MS method which analyzed the VOCs released from the decomposition of human remains (3,19). The National Institute of Standards and Technology (NIST) mass spectral library and extracted ion chromatograms were used to identify the compounds. The first extraction was assessed as day 0 of the experiment. The vials were then opened and maintained at room temperature (22°C) and relative humidity (45%) for a period of 21 days. At the time points of day 3, 7, 14, and 21, headspace extraction was repeated. More precisely, for each time point, vials were closed and had been left in equilibrium for 30 min prior to SPME extraction and subsequent injection.

Dog Training Procedure

For this investigation, during the training and testing sessions, two dogs (dog A: Labrador Retriever, male, 5 years old; dog B: Labrador Retriever, male, 3 years old) worked with their professional dog handlers. These dogs were already established HRD canines, and therefore able to detect human cadaveric blood at the different stages of decomposition. Specifically, we used the same dogs, experimental setup, and training protocol already verified in our previous experiment (29). In order to standardize the environment, it was decided to conduct our testing and training procedure exclusively indoors. The search location was a confined room that had not previously been used for dog detection training purposes. There were five sessions of training and testing, one for each time point of the experiment (precisely at day 0, 3, 7, 14, and 21 from room temperature stabilization). For each session, the vials, containing blood stored samples, were placed in nontransparent glass containers with only one small hole on the top cap. Thus, blood samples were not visible or accessible to the dogs other than by olfaction. These containers were placed on wooden supports and then randomly positioned on the floor. At the same time, we prepared an identical number of empty vials as blank samples. These empty vials were also placed in nontransparent glass containers with only one small hole on the top cap, then posed on wooden supports and randomly positioned on the floor.

Airtight glass containers, rather than plastic containers, were preferred to prevent the smell of plastic in modifying the target odor, in order to prevent the dog looking for the smell of plastic or the smell of the target with the plastic. Latex gloves and disposable coveralls were also used to minimize the spread and the contamination of the target odor.

Each time point of the experiment took place in 5 days and for each one, we measured and recorded the performance of detection dogs in a series of eight tests for each dog (a total of 40 tests for each time point, hence 200 tests for the whole experiment). Our dogs were already trained and therefore able to issue a (sitting) passive alert, without barking, upon detection of the target scent (exclusively human cadaveric blood and not other types of animal blood).

Each session of testing had the same two observers present to obtain a double-blind study: neither handler/dog unit nor observers were aware of the conditions of each search section; they were blind both to the sample (target) positions and to the presence/absence of the target odor. In this way, handlers had to call out the nontransparent glass container suspected of containing the target odor based only on dog choice. To avoid the possibility of mistakes by the handlers (such as rewarding the dogs for a false alert), when a handler "called an alert" the observers recorded the alert location specified by the handler. Observers recorded alerts as called by handlers and did not evaluate the validity of alerts. The experimenter was the only person who was aware of the conditions of each search section. Using a live speaker-phone system, the experimenter, who was isolated, then informed the handlers whether the choice was correct, allowing the handler to reward the dog appropriately. If the response was a correct detection, it was classified as a true positive (TP); and the dogs were requested to continue sniffing all the holes placed in the testing arena. Whereas, if the response was a false alarm (the target was not present and the dog reported it was present), it was classified as a false positive (FP). A false negative (FN) was recorded when the target was not signaled by the dogs. In these cases, only for training purposes, the trial was repeated to refresh the memory of the dog until the dog performed without any misses or false alarms for five consecutive trials. A true negative (TN) was recorded when the target odor was not present and the dogs did not issue any alerts. A true negative was recorded when the target was not present and the dogs did not issue any alerts.

Results and Discussion

HS-SPME/GS-MS Analysis

The VOCs being released by each specimen were extracted and analyzed using HS-SPME/GC-MS; we obtained a long list of compounds including acids, alcohols, aldehydes, aromatic hydrocarbons, halogens, and ketones (31). Only VOCs that had been previously cited in the literature as originating from human specimens were used in the analysis of our samples(3,14–16,18,19,28,32–36). VOCs detected by blood samples, both fresh and decomposed, are summarized in Table 1.

We did not find any differences between white and black corpses. Qualitative differences were observed in the headspace VOCs composition across the five-time points analyzed. As previously described, our findings demonstrated that, although different decompositional stages have been identified, the chemical changes of decomposition occur on a continuum (14,20,37–39). The VOCs profile was obtained from fresh to decomposed blood samples, showing differences in the presence of specific compounds, although within the same compound classes.

In some studies, it was shown that changes in VOCs can also be caused by bacterial metabolic processes (40,41).

Hoffman et al. described that they did not identify skatole in the headspace of any of their samples (19).

Indole, tetrachloroethylene and hexanoic acid ethyl ester were detected both at day 0 and day 3; at the other time points, the aromatic heterocyclic compounds, the halogenated hydrocarbons, and the esters disappeared. Hydrocarbons (toluene, decane, and hexane) were detected at day 7. As time progressed (day 14 and day 21), only straight chain hydrocarbon hexane was detected. Aldehydes were identified in all the samples. The blood at days 0 and 3 contained benzaldehyde and 1-Octanal, which disappeared at days 7, 14, and 21 of the experiment when other aldehydes were present in the headspace: nonanal and heptanal. Acetaldehyde appeared at days 14 and 21. Acids (propanoic, butanoic, and hexanoic) were present in the headspace at day 0 and day 3, while they disappeared from headspace at the following time points (day 7, 14, and 21). Ketones were present in all samples with a different profile: 2-butanone at day 0 and day 3; cyclohexanone at the other time points. Alcohols (1-Pentanol, 2-Methylbutanol) were detected in the headspace at day 0 and day 3; they disappeared at day 7. At day 14 and day 21, ethyl alcohol was detected in the headspace.

In fresh blood, Chilcote et al. (42) identified seven compounds characterized by high discriminatory power: 2,4,4-trimethyl-1-pentene, 3-methyl-6-(1-methylethyl)-2-cyclohexen-1-one, 5-methyl-2-hexanone, 2-nonanone, 2,2,4-trimethyl-3-penten-1-ol, aniline and methylcyclopentane. Over days from 1 to 22, 2-pentylfuran, heptanal, and nonanal prevailed as discriminatory trait of blood samples. Similarly, our results demonstrated forward decompositional progresses, the more heptanal and nonanal were present.

However, to better study human cadaveric decomposition olfactive signatures, previous studies were compared, even if conducted, above all, on animal models in order to mimic human decomposition. In a murine model as human equivalent, 18 VOCs were identified, among which butanoic acid, 3-methyl butanoic acid, pentanoic acid, hexanoic acid, and indole were present at each sampling time (40). Agapiou et al. (43) on a pig model, as human surrogate, identified "scent of death" as 288 VOCs, among which the majority were aliphatics, aromatics, and nitrogen categories, followed by less representative compounds (ketones, esters, alcohols, aldehydes, sulfur, miscellaneous, and acid compounds). Rosier et al. (44), instead, studied pig remains as human analogue and identified eight specific VOCs (ethyl propionate, propyl propionate, propyl butyrate, ethyl pentanoate, pyridine, diethyl disulfide, methyl(methylthio) ethyl disulfide, and 3-methylthio-1-propanol). Forbes et al., moreover, studied how the decomposition VOC profile of pig carcasses changed across the seasons, in relation to the different decomposition progression: resulting in the reduced VOC profile in winter according to the slower rate of decomposition. However, both in winter and summer the amount of VOCs increased rapidly during active decay, followed by a decrease in the advanced decay phases and, finally, another increase phase during the subsequent decomposition steps. In both cases, sulfur-containing compounds, ketones, and alcohols were the most common VOC categories, with sulfur-containing compounds most characteristic of the summer VOC profile. On the other hand, ketones and alcohols were more prominent during active and advanced decay stages while hydrocarbons were discriminatory during the dry stage (45). Recently, DeGreeff and Furton studied 27 deceased individuals and isolated 12 VOCs determined to be significant to the odor profile of human remains (22). In the same study, a VOC profile comparison between living and deceased individuals showed intersubject differences in

TABLE 1—*List of compounds recorded by blood samples, both fresh and decomposed.*

	Day 0	Day 3	Day 7	Day 14	Day 21
Acids	Propanoic acid Butanoic acid Hexanoic acid	Propanoic acid Butanoic acid Hexanoic acid			
Sulfides			Dimethyl Disulfide	Dimethyl Disulfide Carbon Disulfide	Dimethyl Disulfide Carbon Disulfide
Ketones	2-Butanone	2-Butanone	Cyclohexanone	Cyclohexanone	Cyclohexanone
Alcohols	1-Pentanol 2-Methyl butanol	1-Pentanol 2-Methyl butanol		Ethyl alcohol	Ethyl alcohol
Esters	Hexanoic acid ethyl ester	Hexanoic acid ethyl ester			
Aldehydes	Benzaldehyde 1-Octanal	Benzaldehyde 1-Octanal	Heptanal Nonanal	Heptanal Nonanal Acetaldehyde	Heptanal Nonanal Acetaldehyde
Halogenated hydrocarbons	Tetrachloroethylene	Tetrachloroethylene			
Hydrocarbons			Toluene Decane Hexane	Hexane	Hexane
Aromatic heterocyclic compounds	Indole	Indole			

the first group. Instead, odor varied less in the second group, suggesting a sort of uniform odor among human remains. Instead, human samples presented lower levels of nitrogen and sulfide volatile compounds compared to swine while the aromatics are mostly represented. Hydrocarbons seem to be similarly released by pig and human decomposition. These data are of interest for the identification of human decomposition VOC signature (46).

In our study, consistently with the studies reported, VOCs changed over time, even when, as in our experiment, environmental conditions were held constant. No qualitative differences were observed between blood samples at day 0 and day 3. When analyzing the decomposing samples, acids, esters, aromatic heterocyclic compounds, and halogenated hydrocarbons disappeared; hydrocarbons appeared and aldehydes relatively became more numerous. As time progressed, sulfides were detected and

TABLE 2—*The overall detection performance of the two dogs across the different time point samples.*

Time point	Trials	Positive	True Positive	False Positive	PPV	Negative	True Negative	False Negative	NPV
Dog A									
Day 0	200	194	193	1	99.48%	6	6	0	100.00%
Day 3	200	192	190	2	98.96%	8	6	2	75.00%
Day 7	200	192	191	1	99.48%	8	7	1	87.50%
Day 14	200	196	195	1	99.49%	4	4	0	100.00%
Day 21	200	199	199	0	100.00%	1	1	0	100.00%
Dog B									
Day 0	200	188	187	1	99.47%	12	11	1	91.67%
Day 3	200	191	190	1	99.48%	9	8	1	88.89%
Day 7	200	193	192	1	99.48%	7	7	0	100.00%
Day 14	200	196	195	1	99.49%	4	4	0	100.00%
Day 21	200	200	200	0	100.00%	0	0	0	–

PPV = TP/(TP + FP); NPV = TN/(TN + FN).

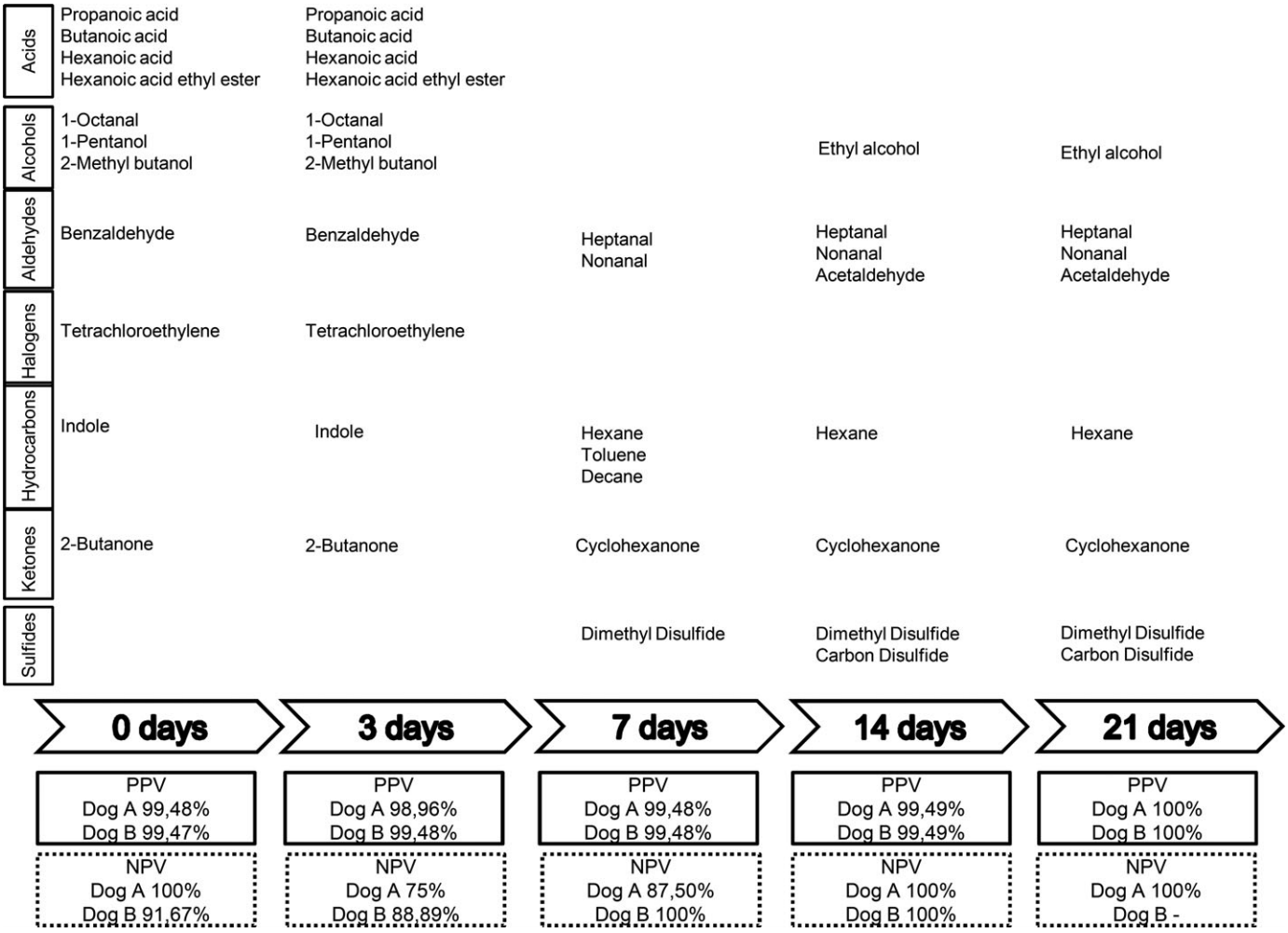

FIG. 1—*Summary of the animal performance over the decay blood process.*

compounds such as dimethyl disulfide and carbon disulfide, commonly associated with decompositional events, appeared. Alcohols are qualitatively more represented in fresh samples rather than decomposed samples and their profile changed over time: ethanol, while not detected in the initial decomposing phases, appeared at day 14 and day 21.

Dog Performance

For each training session, the PPV (the proportion of the canine target—present responses that are correct) and the NPV (the proportion of the canine target—absent responses that are correct) were calculated. These two parameters express how predictive is the dog, indicating the likelihood of target presence if the dog indicates the target is present and the likelihood that a target is absent if the dog does not indicate the target (47). Table 2 summarizes the overall detection performance of the two dogs across the different time point samples.

The positive predictive value (PPV) ranged between 98.96% and 100% for *DOG A*, between 99.47% and 100% for *DOG B*; the negative predictive value (NPV) was measured between 75% and 100% for *DOG A*, between 88.89% and 100% for *DOG B* (Fig. 1).

As expected, at day 0 (fresh, undiluted human cadaveric blood), both dogs showed good performance rates (29). Moving from day 3 toward more advanced phases of the blood decomposition process, we noted an improvement in both animal performances that reached the highest levels at day 14 and day 21.

One possible explanation could be related to the VOCs found in the advanced decomposition phases which sufficiently differ from those found in earlier blood decomposition. Indeed, during these lapses, sulfur compounds (dimethyldisulfide and carbon disulfide) were detected by GC-MS. It is well known that the mammalian olfactory receptor is a metalloprotein and that metallic zinc nanoparticles could be involved in signal transduction between the receptor proteins and the heterotrimeric guanine nucleotide-binding proteins (G-proteins) and the effector enzymes that are activated by odorant binding (48–50). Sulfur compounds bind strongly to zinc (50).

Since there is no reason to believe that relative affinities are systematically different between dogs and humans, this may explain the better performance rates of our dogs when sulfur compounds were present in the headspace (49). Another point of interest is the fact that the performance of both dogs showed an increasing trend from day 14 to day 21, although the VOCs pattern appeared qualitatively the same. A hypothesis is that a quantitative difference, perhaps in sulfur compounds that are very potent odors, would allow for the better performance of the dogs. Furthermore, the effect of continuous training on the dog's ability to alert is not negligible. In fact, as explained in the "Dog Training Procedure" our dogs were already established HRD canines; and so, they were also able to detect an alert human cadaveric blood in different stages of decomposition. Moreover, the repeated exposure to a specific odor improves the dogs' ability to detect and alert the same (smell memory in the midbrain) (51–54).

Conclusions

The odor signature of decomposing animal and human remains that canines recognize as target odor is still poorly understood so that the choice of the optimal human tissue for training dogs is still debated (19).

Moreover, while the use of canines as a specialized biological device in identifying human remains has been accepted over the past decades, the instrumental detection of human decompositional VOCs as a form of forensic evidence still remains a challenge.

This study explored the analytical technique (GC-MS) and the olfactory testing of dogs in order to: first, identify which VOCs are detected by GC-MS from human blood decomposition and how these changed over time and second, demonstrate the ability of trained canines to detect human blood decomposition scent and precisely locate the target odor emanation point.

Our findings demonstrated that decomposing human blood is a good source of VOCs and in parallel is a good target capable to elicit the canine olfactory response in well-trained canines. Overall, our results demonstrate that GC-MS, combined with experimental field tests using trained dogs, could be an effective method for determining the appropriate odor chemicals required to train canines and for identifying active odor signature chemicals in forensic specimens.

Our study confirms the reliability of well-trained dogs as specialized biological devices in revealing blood presence, under the conditions described in this manuscript. This behavior has lead us to hypothesize that with a standardized protocol, these dogs will be able to specifically locate blood traces covering large distances in a relatively short period and not of other biological material in order to allow for the subsequent confirmative test. This would be useful in all cases where it would be necessary to identify and then isolate a crime scene in which bloodshed occurred: murders, even with occulted cadavers, assault, injury.

However, further research is required in order to investigate both the true capability of dogs in "real work" field scenarios and a better characterization of the volatile odor signature.

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