Quantification of brown dog tick repellent, 2-hexanone and benzaldehyde, release and from tick-resistant Beagle dogs, *Canis lupus familiaris*

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Abstract

We have recently shown that repellency of the tick *Rhipicephalus sanguineus* sensu lato by the tick resistant dog breed beagle is mediated by volatile organic compounds 2-hexanone and benzaldehyde present in beagle dog odour. Ectoparasite location of animal hosts is affected by variation in odour components and ratios. The aim of this study was to develop an analytical method to quantify the release rate, and the ratio, of 2-hexanone and benzaldehyde from beagle dogs. The odour of three beagles was collected, for four days, over one week (day 0, day 1, day 4 and day 7). The compounds were identified using coupled high-resolution gas chromatography–mass spectrometry (GC-MS), and authentic standards of compounds were used to generate external calibration curves for quantification. Both compounds were found in all dogs on all days. The amount of benzaldehyde was always higher than that of 2-hexanone and so their ratio varied from unity, on average (over time) being 3.128 ± 0.365 , 1.902 ± 0.390 , 1.670 ± 0.671 ng.mL⁻¹ for beagle 1, 2 and 3, respectively. There was no significant ($p < 0.05$, F-test) effect of time. The overall mean was 2.233 ± 0.387 ng.mL⁻¹. Our results enhance our previous findings by documenting the presence of 2-hexanone and benzaldehyde in beagle dog odour samples covering a 7-day period. This knowledge should support the development of slow-release formulations to protect dogs from *R. sanguineus* s. l. infestation.

Keywords: 2-hexanone; Benzaldehyde; VOC's; *Rhipicephalus sanguineus* sensu lato; Non-host; GC-MS;

1. Introduction

Odour chemicals produced by animals can affect the behavior of another species [1,2]. In particular, repellency and attraction are two important interspecific olfactory effects that occur during the interactions between ticks and their hosts [3-6]. Ticks can use chemical cues to find hosts, aggregate on a host, and mate, so the discovery of repellents that can disturb these processes is an important step towards developing new control technologies that could also minimize the risk of tick-borne disease transmission to human and domestic animals through the bites of infected ticks. [7,8].

The brown dog tick, *Rhipicephalus sanguineus* sensu lato, is a serious pest of concern to public health and also of veterinary importance for animals at risk [9-12]. *R. sanguineus* utilizes volatile semiochemicals present in breath, skin of its hosts as a mode of host location [13,14]. Louly et al. [15] demonstrated that a higher number of *R. sanguineus* s. l. ticks were recovered in English cocker spaniel than beagles, and later demonstrated that *R. sanguineus* s. l. uses chemoreception when choosing between either resistant (beagle), or susceptible (English cocker spaniel), dogs as hosts. In olfactometer tests, comparing the odours from both breeds, ticks were observed to avoid the odour of beagles. This avoidance behaviour elicited by resistant dogs was found to be associated with two compounds, 2-hexanone and benzaldehyde, found in their odours, which act as natural tick repellents [14]. When these two compounds were presented together in a 1:1 ratio, the repellency observed was higher than when they were tested alone, and it was similar to or even higher than the repellency observed using DEET (N,N-diethyl-3-methylbenzamide), a standard repellent.

Alternatives to the common use of synthetic acaricides are necessary because of side effects such as environmental pollution and especially the contamination of animal products [16]. There is also the risk of emergence of tick resistance to acaricides [17-20]. A desirable approach is the integration of biological technologies and volatile compounds that alter tick

behavior [21]. Borges et al. [14] found compounds in resistant beagle dogs that could be used in such an approach. However, it has been observed in resistant and susceptible hosts that the naturally occurring association of chemical compounds can blind, mask, or expand the perception of other chemical compounds acting as signals to parasites [22-24]. Thus, the combination, or, in mathematical terms, the ratio of compounds in each host can act synergistically or antagonistically against parasites [22,23,25-27]. Therefore, the aim of this study was to analyze and quantify the release rate, and the ratio of 2-cyclohexanone and benzaldehyde odor in beagle dogs.

2. Material and Methods

2.1. Odour collection

Odour components from one male and two female beagle dogs (all 8 years old) were collected by adapting techniques previously described [28]. Briefly, odour components were adsorbed in a mix of activated charcoal (350 mg, 40 µm; Fluka, Sigma-Aldrich) and octadecyl bonded silica (450 mg, Bakerbond®, C18, particle size 40 µm; JT Baker-Hexis, Sigma-Aldrich) that was packed in a piece of filter paper (9 x 8 cm piece of Whatman no. 1 qualitative) stapled securely to produce an adsorbent sachet (4 x 4 cm). Each adsorbent sachet was encased in a folded piece of stainless steel mesh-wire sheet (100 µm; 5 x 10 cm) that stapled securely together. A piece of aluminum foil was used to cover each odour trap. Two odour traps were sown with nylon fishing line to the inner side of a strip of elastic material with Velcro at each end. These strips formed collars for the dogs, fitted with the aluminum covered odour traps, and they were kept at -20°C until use. The aluminum was removed from the exposed side of each odour trap before the collar was placed on the dog's neck. A different odour trap collar was used every time a dog was sampled for 7h. A total of four samples were obtained from each dog, at 1, 4, and 7 days after the initial sample was collected on day 0. After each sampling, odour trap collars were stored in a freezer at -20 °C until extraction. The adsorbent mix from two sachets in each of the 12 odour trap collars

collected was pooled, and transferred to an Octadecylsilane (ODS) cartridge (Accu Bond solid-phase extraction; J&W Scientific), eluted with 4 mL of dichloromethane (DCM) (HPLC> 99.8%, Sigma Aldrich), concentrated to 150 μ L under a gentle stream of clean nitrogen, and the extract bottled in an amber ampoule for storage at -20 °C.

2.2. Gas chromatography (GC) and coupled GC-mass spectrometry (GC-MS) analysis.

Extracts (1 μ L) were analysed on an Agilent 6890A GC (Agilent Technologies, Santa Clara, California, USA), equipped with a cool column injector, flame ionization detector (FID), and a 50 m $\times$ 0.32 mm i.d. HP-1 column. The oven temperature was maintained at 30 °C for 0.1 min, then programmed to increase at 10°C.min⁻¹ until 250°C, and then held for 38 min. For GC/MS (VG Autospec Ultima, Manchester, UK) compound identification, an HP-1 capillary GC column (50 m $\times$ 0.32 mm i.d. $\times$ 0.52 μ m film thickness), equipped with a cool on-column injector was used. Ionization was accomplished by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for five minutes and then programmed to increase 5°C.min⁻¹ until 250°C. The carrier gas was helium. Tentative identification by GC/MS was confirmed by comparing retention indices of peaks with those of authentic standards and by peak enhancement on GC by co-injection with authentic compounds, using an Agilent 6890N GC with 10 m $\times$ 0.32 mm i.d. HP-1 column. The oven temperature was maintained at 30°C for 0.1 min, then programmed to increase at 10°C.min⁻¹ until 250 °C, and then held for 30 min.

2.3. VOC's (2-hexanone and benzaldehyde) quantification

Analytical curves were constructed for determination of the concentration of 2-hexanone and benzaldehyde in the dog odour extracts. Primary stock standard solutions of 2-hexanone (Aldrich Chemical Co. Ltd., Dorset, UK) and benzaldehyde (Sigma-Aldrich Co. Ltd., Steinheim, Germany) at a concentration of 1000 ng.mL^{-1} were prepared using DCM bi-distillate (Sigma-Aldrich Co., St. Louis, Missouri, USA) as solvent. Stock solutions were diluted serially to reach a concentration of 10 ng.mL^{-1} , which was used to produce five different concentrations of 2-hexanone (2.5, 1.5, 1.0, 0.5 and 0.1 ng.mL^{-1}) and benzaldehyde (6.0, 5.0, 4.0, 2.5 and 1.0 ng.mL^{-1}). Each final concentration was tested in triplicate. One μl of each solution was injected and analysed by GC-FID using the method as shown in the previous section. This method was adopted to account for technical variation in the calibration procedure. Goodness of fit of the calibration curves was determined by linear regression analysis with the chromatographic areas (mAU) obtained from the GC being the response variable, y , and the corresponding concentrations injected to secure those areas, of either 2-hexanone or benzaldehyde compounds (ng.mL^{-1}), being the explanatory variable, x . Using the range of concentrations of each compound, the linear regression model was $y = c + bx$, where c is the intercept and b is the slope of the fitted line. The strength of the relationship in each case was judged in terms of the proportion of variation explained, R^2 , and the significance ($p < 0.05$) of the F-test from the analysis of variance (ANOVA) accompanying the regression. Evidence of any curvature was assessed by adding a quadratic term in concentration into the linear model and testing the improvement in fit (F-test). The model was fit using ordinary least squares, which provides estimates of the parameters c and b along with standard errors. The linear formulae obtained from this analysis were used to determine the concentration of each compound in each sample, by substituting in the values of areas, y , for the samples, to calculate the corresponding concentrations, x . Ratios were obtained by dividing the quantity of benzaldehyde by 2-hexanone collected every day from each dog. ANOVA was applied to the concentration data for each compound and the ratio, to assess the significance ($p < 0.05$, F-tests) of overall difference between beagles and between time points, noting the four repeated measures

from each beagle. The limit of detection (LOD) and the limit of quantification (LOQ) were determined in accordance with the linear regression, and are expressed as:

$$\text{LOD} = 3.3 \times \text{SE}(c)/b$$

$$\text{LOQ} = 10 \times \text{SE}(c)/b$$

where: $\text{SE}(c)$ is the standard error of the intercept and b is the slope of the analytical curve.

The GenStat (17th edition, © VSN International Ltd, Hemel Hempstead, UK) statistical package was used for analysis. No transformation of the data was required, inspection of residuals confirming that the data broadly complied with the assumptions of the analysis.

3. Results

3.1. Quantitative analysis of tick repellent compounds

The presence of 2-hexanone and benzaldehyde in the odour samples was confirmed by direct comparison of their mass spectra and retention times with those of known concentrations of authentic standards using the co-injection GC-FID method. Co-injection of Beagle odor extracts with authentic standards 2-hexanone (0.033 ng.mL⁻¹) and benzaldehyde (0.016 ng.mL⁻¹) gave a clear result, with the peaks in the standards relating to corresponding peaks for the dogs. A first peak was identified as 2-hexanone and a second as benzaldehyde (Fig. 1). The full set of 12 spectra (three dogs by four time points) is given in Fig. 2. The two compounds were quantified (Table 1) by using the fitted analytical calibration curves. Table 2 gives details of data for the ratio of the two compounds. Over the concentration range of 0.1 – 2.5 ng.mL⁻¹ for 2-hexanone, and of 1.0 – 6.0 ng.mL⁻¹ for benzaldehyde, there was a linear relationship for the compounds. There was a strong correlation between the concentrations of the compounds in the dilution series of the standard samples with the corresponding peak area in the chromatograms. Table 3 and 4 show the ANOVA output of the linear regression models for 2-hexanone and for benzaldehyde along with the estimated parameters (intercepts and slopes) in the models.

For 2-hexanone, there was a highly significant regression ($p < 0.001$, F-test) and also for benzaldehyde ($p < 0.001$, F-test). For the latter compound the intercept value was not statistically different from zero ($p > 0.05$, F-test) and there was no evidence ($p < 0.05$, F-test) of curvature for either compound 2-hexanone ($p = 0.822$, F-test) or for benzaldehyde ($p = 0.207$, F-test). The adequacy of the linear model was also assessed in terms of the very high proportion of variance explained (R^2), being 0.9827 for 2-hexanone and 0.9948 for benzaldehyde. being 98.27% for 2-hexanone and 99.48% for benzaldehyde.

3.2. *Estimation of the quantity of 2-hexanone and benzaldehyde produced by the Beagle dogs*

The range in concentration of 2-hexanone in the odour from beagles was 0.633 ng.mL^{-1} in beagle 1 on the 7th day to 3.405 ng.mL^{-1} in beagle 3 on the 7th day (Table 1). For benzaldehyde, the range observed was 1.479 ng.mL^{-1} in beagle 1 on the 4th day to 6.038 ng.mL^{-1} in beagle 2 on the 4th day. The ratio of benzaldehyde:2-hexanone varied from 1.135 to 3.691, being, on average (over time), 3.128 ± 0.365 , 1.902 ± 0.390 , $1.670 \pm 0.671 \text{ ng.mL}^{-1}$ for beagle 1, 2 and 3, respectively. The overall average was $2.233 \pm 0.387 \text{ ng.mL}^{-1}$. ANOVA revealed no significant effect of time points ($p < 0.05$, F-tests) for either of the compounds or the ratio. There was some evidence of real difference ($p = 0.057$, F-test) between beagles for the ratio, this evidence clearly being derived from the effect of beagle for 2-hexanone ($p = 0.096$, F-test) rather than benzaldehyde ($p = 0.563$, F-test). Beagle 1 had a far greater ratio than the other two beagles (Table 2), due to much lower mean concentrations of 2-hexanone over time (Table 1). For 2-hexanone the LOD and the LOQ were found to be 0.092 and 0.278 ng mL^{-1} respectively and for benzaldehyde the LOD and the LOQ were found to be 0.270 and 0.819 ng mL^{-1} respectively. For benzaldehyde the LOQ was smaller than the first point of the calibration curve ($\leq 1.00 \text{ ng mL}^{-1}$) indicating that, assuming linearity, smaller quantities than the smallest calibrated could be estimated from corresponding

chromatographic areas, although for robust estimates from the regression analysis it would still be advisable to remain within the limits of the calibration.

4. Discussion

Here, we report the development and validation of an analytical method to quantify the relatively minor amounts of the tick repellents 2-hexanone and benzaldehyde in the odour of beagle dogs in a consistent and reliable manner and hence also the natural ratio of benzaldehyde to 2-hexanone in the odour. Our results enhance previous findings [14] by documenting the presence of 2-hexanone and benzaldehyde in beagle dog odor samples covering a 7-day period. Previous studies showed that the beagle is a dog breed resistant to tick infestation and that repellency is associated with host odor components [14,29]. Although the 1:1 combination of 2-hexanone and benzaldehyde was shown to be as repellent as DEET, the *in vivo* variation in their concentrations over time was not documented. The actual benzaldehyde:2-hexanone ratio, based on quantitative readings in the current study, ranged from 1.1:1 to 3.7:1, average 2.2:1, which suggests there is most usually around 2-fold greater benzaldehyde than 2-hexanone in the natural repellent. To our knowledge, this is the first study that quantifies these repellent compounds, produced naturally by animals, for a period longer than one day in order to document their persistence in proportions shown to repel ticks *in vivo* and *in vitro*. A 2:1 benzaldehyde:2-hexanone ratio should be tested in future experiments.

Odour compounds can be specific to particular animals and function as chemical signatures from an ecological perspective [14,28,30,32,34]. VOCs acquired and developed during life are influenced by the physiological state of living organisms [31,32,33,35-38]. Plant VOCs can change daily and weekly [27,39]. The isomers 4aS,7S,7aR-, and 4aS,7S,7aS-nepetalactone in the essential oil of *Nepeta cataria* (Lamiaceae), which confer tick repellency, varied during the course of ten weeks [40]; however, the quantitative and

proportional fluctuation of the compounds across time was not reflected in variation of efficacy. Remarkably, the analytical results for 2-hexanone and benzaldehyde reported here and obtained from older beagles are comparable to those reported in younger dogs [14].

An important branch of chemical ecology research is the innovation and adaptation of technologies to determine the ratio of repellent compounds in biological matrices. Parasitism perhaps drives the evolution of repellent mixtures produced by hosts, and the actual mixture composition and proportion of its components may be linked to host susceptibility or resistance [22,23,25-27]. Knowledge of the ratio of principal bioactive components in natural mixtures with repellent effects also facilitates the development of formulations to protect humans or domestic animals. As previously done by others documenting the importance of elucidating the ratio of repellents in natural substances to inform formulation [27,31], we showed that benzaldehyde is relatively more abundant than 2-hexanone in beagle odor and suggest that a 2:1 benzaldehyde:2-hexanone ratio should be used to develop and test sustained release formulations.

The cosmopolitan brown dog tick, *R. sanguineus* s. l., is one of the species making the global problem of ticks and tick-borne diseases more complex [20,41,42]. Developing strategies that integrate diverse technologies is an approach that will help manage ticks, and the pathogens they transmit, in a sustainable manner [43,44]. The use of anti-tick vaccines has been proposed as a way to protect dogs from infestation and tick-borne disease transmission [44,45]. Our findings provide additional supportive information for the development of a formulation combining benzaldehyde and 2-hexanone to protect dogs susceptible to infestation with *R. sanguineus* s. l. [14]. Integrated treatment, in areas where there are tick infestations, that combines anti-tick vaccination with the use of a formulation based on repellents discovered in beagle dogs would protect not only dogs, but also humans from tick parasitism and the zoonotic pathogens *R. sanguineus* s. l. transmits.

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Figure and tables legends.

Figure 1. Co-injection of Beagle odour extracts with authentic standards 2-hexanone (0.033 ng.mL^{-1}) and benzaldehyde (0.016 ng.mL^{-1}); A: synthetic compounds; B: Dichloromethane extract from beagle 1 day 0; C: Co-injection of beagle sample with synthetic 2-hexanone and benzaldehyde. The peak for 2-hexanone is labelled as 1; the peak for benzaldehyde is labelled as 2.

Figure 2. The GC and GC/MS of DCM extracts of the odours of the three beagles, in all four days evaluated. The 12 spectra were highly similar, and 2-hexanone and benzaldehyde were present in all extracts. A1: Beagle 1 day 0; A2: Beagle 1 day 1; A3: Beagle day 4; A4: Beagle day 7; B1: Beagle 2 day 0; B2: Beagle 2 day 1; B3: Beagle 2 day 4; B4: Beagle 2 day 7; C1: Beagle 3 day 0; C2: Beagle 3 day 1; C3: Beagle 3 day 4; C4: Beagle 3 day 7; the peak for 2-hexanone is indicated labelled as 1; and the peak for Benzaldehyde is labelled as 2.

Table 1. Concentration of 2-hexanone and benzaldehyde in odour samples of three beagles across four days during one week, having analyzed samples by GC and conversion of peak areas from the spectra into concentrations (ng.mL^{-1}) via linear regression models (see Tables 3 and 4). Beagles 1 and 3 are female, beagle 2 is male. There were no significant ($p < 0.05$, F-tests) effects of time or between beagles.

Table 2. Ratio of benzaldehyde to 2-hexanone in odour samples of three beagles across four days during one week. Beagles 1 and 3 are female, beagle 2 is male. There was some evidence of real difference between beagles ($p = 0.057$, F-test), the standard error of the difference between the means for the beagles was 0.506 on 6 degrees of freedom.

Table 3. Summary of the output of the ANOVA for the linear regression analysis of 2-hexanone. The degrees of freedom (df), sums of squares (SS), mean squares (MS), variance ratio (VR) and p-value for the F-test of the variance ratio (F.pr.) are given along with the estimated coefficients in the linear regression model, their standard errors (SE), partial t-statistics on 13 df and p-values for the t-tests of these t-statistics.

Table 4. Summary of the output of the ANOVA for the linear regression analysis of benzaldehyde. The degrees of freedom (df), sums of squares (SS), mean squares (MS), variance ratio (VR) and p-value for the F-test of the variance ratio (F.pr.) are given along with the estimated coefficients in the linear regression model, their standard errors (SE), partial t-statistics on 13 df and p-values for the t-tests of these t-statistics.

Figure 1

Figure 2

Table 1

2-Hexanone							Benzaldehyde					
Beagle	Day 0	Day 1	Day 4	Day 7	Mean	SE	Day 0	Day 1	Day 4	Day 7	Mean	SE
1	0.652	0.717	0.662	0.633	0.666	0.021	2.407	2.278	1.479	2.157	2.080	0.239
2	1.586	1.150	2.157	1.175	1.517	0.272	2.364	1.480	6.038	2.386	3.067	1.169
3	1.329	1.919	1.305	3.405	1.989	0.569	1.961	3.545	2.900	3.865	3.068	0.485
Mean	1.189	1.262	1.374	1.738	1.391		2.244	2.434	3.472	2.802	2.738	
SE	0.227	0.287	0.354	0.693	0.269		0.116	0.491	1.100	0.437	0.421	

Table 2

Beagle	Day 0	Day 1	Day 4	Day 7	Mean	SE
1	3.691	3.176	2.235	3.410	3.128	0.365
2	1.491	1.286	2.800	2.030	1.902	0.390
3	1.475	1.848	2.223	1.135	1.670	0.271
Mean	2.219	2.103	2.419	2.192	2.233	
SE	0.736	0.560	0.190	0.662	0.287	

Table 3

	df	SS	MS	VR	F.pr.
Regression	1	3874.42	3874.42		
Residual	13	19.91	1.531	2530.14	<0.001
Total	14	3894.33	278.17		
Percentage variance accounted for (R^2) 99.4					
Standard error of observations is estimated to be 1.24					
	Coefficients	SE	t (13)	T pr.	
Constant (<i>c</i>)	0.289	0.534	0.54	0.598	
Concentration (<i>b</i>)	19.242	0.383	50.30	< 0.001	

Table 4

	df	SS	MS	VR	F.pr.
Regression	1	5481.96	5484.963	2508.86	< 0.001
Residual	13	28.41	2.185		
Total	14	5510.37	393.598		
Percentage variance accounted for (R^2) 99.4					
Standard error of observations is estimated to be 1.48					
	Coefficients	SE	t (13)	t pr.	
Constant (c)	-0.981	0.881	-1.11	0.286	
Concentration (b)	10.754	0.215	50.09	< 0.001	