



# TECHNICAL NOTE: Vapor release of 2-methyl-2-butenal from dog collars and spray containing pheromone or interomone measured by solid-phase microextraction and gas chromatography-mass spectrometry

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## ABSTRACT

The molecule 2-methyl-2-butenal (2M2B) is a maternal pheromone in rabbits and an interomone in dogs eliciting behavioral and heart-rate changes. The molecule 2M2B is manufactured in dog collars (Nurturecalm 24/7 Canine Calming Pheromone Collar, Meridian Animal Health, Omaha, NE) and in aerosol sprays (Sentry Calming Spray for Dogs, Sergeant's Pet Care Products Inc., Omaha, NE). To determine volatile release of this molecule in the collar and liquid, solid-phase microextraction

followed by gas chromatography-mass spectrometry was performed on the headspace over the test materials. In Exp. 1, a snippet of dog collar and 3 mL of aerosol liquid were compared with a standard of 2M2B in isopropyl alcohol. In Exp. 2, a dog collar was removed from its wrapping and placed at room temperature for 49 d. On d 0, 7, 14, 21, 28, 35, 42, and 49 relative concentrations of molecules volatilized were calculated from area under the corresponding gas-chromatography peak. Release of 2M2B followed an exponential decay rate over time. In Exp. 3, collars were placed on 3 dogs for 35 d. Dogs were variable in their level of activity. Dog-worn collars had smaller and more variable concentrations of the 2M2B in the head space at 35 d than the same collar kept in the laboratory at room temperature. This paper

authenticates a technique for the assay of low concentrations of pheromones or interomones released from plastic collars or a liquid formulation. Furthermore, pheromone or interomone release over time was documented both in laboratory and field settings.

**Key words:** interomone, 2-methyl-2-butenal, dog, solid-phase microextraction, pheromone

## INTRODUCTION

The molecule 2-methyl-2-butenal (2M2B), a pheromone found in the milk of the European rabbit, *Oryctolagus cuniculus*, initiates an innate attraction and oral grasping response in pups (Schaal et al., 2003). Maternal pheromones in mammalian species

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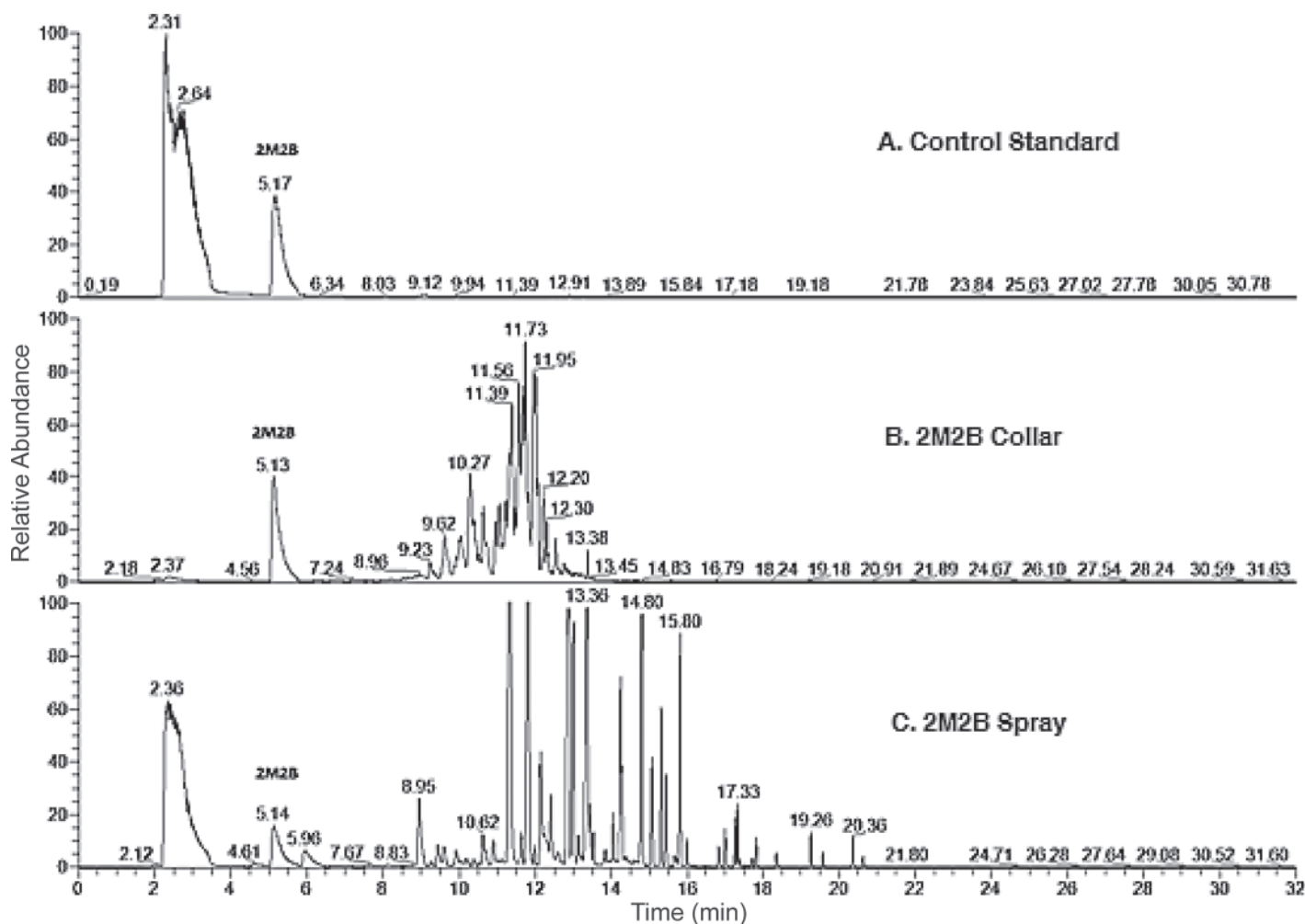
aid in newborn survival by helping animals find a food source, creating a parental-offspring bond, or decreasing aggression (Leon and Moltz, 1971; Morrow-Tesch and McGlone, 1990a,b; Pageat and Teissier, 1998; Pageat, 2001). Maternal pheromones have unique effects with adult members of the same species and in some cases calm them (Sheppard and Mills, 2003; Falewee et al., 2006). Pheromones, including maternal pheromones, can also have unique effects in other species, suggesting that multiple species use similar chemical messengers (Wyatt, 2003).

The term "interomone" was created to classify the increasing number of molecules that act as pheromones

in one species and change different behaviors in other species. One interomone naturally produced in swine, androstenone, was shown to decrease barking and jumping behavior in dogs (McGlone et al., 2014). The molecule 2M2B may also act as an interomone by changing heart rate and behavior in dogs (J. J. McGlone, unpublished data). The 2M2B molecule is manufactured in collars and sprays sold by Sergeant's Pet Care Products Inc. (Omaha, NE) and Meridian Health (Omaha, NE). Because of the complexities of the manufacturing process, it is necessary to verify that the molecule is present in the air around the collar on dogs and for some period of time after the package is opened.

Solid-phase microextraction (SPME) analyzed with gas chromatography-mass spectrometry (GC-MS) was used to detect the presence of 2M2B. This method is highly sensitive (Górecki et al., 1999; Koziel et al., 1999), but its use to demonstrate pheromone release of 2M2B has not been previously published.

The first experiment determined whether 2M2B could be detected in the head space around a collar and over the liquid. The second experiment determined the rate at which 2M2B volatilizes at room temperature over 49 d. The third experiment determined the effects of dog activity on the amount of 2M2B volatilized over 35 d.



**Figure 1.** The gas-chromatography traces of solid-phase-microextraction analysis for the molecule 2-methyl-2-butenal (2M2B) in the immediate air surrounding samples. (A) 2M2B in isopropyl alcohol control standard, (B) 2M2B dog collar, (C) 2M2B aerosol spray liquid. Isopropyl alcohol was present at 2.36 to 2.80 min in both the control standard and the aerosol spray liquid acting as a transport medium for 2M2B. 2-Methyl-2-butenal was present at 5.13 to 5.20 min. Synthetic lavender fragrance molecules were present in both the 2M2B collar and spray liquid at 7.24 to 22.81 min.

## MATERIALS AND METHODS

### Exp. 1: 2M2B Release from Collar and Liquid

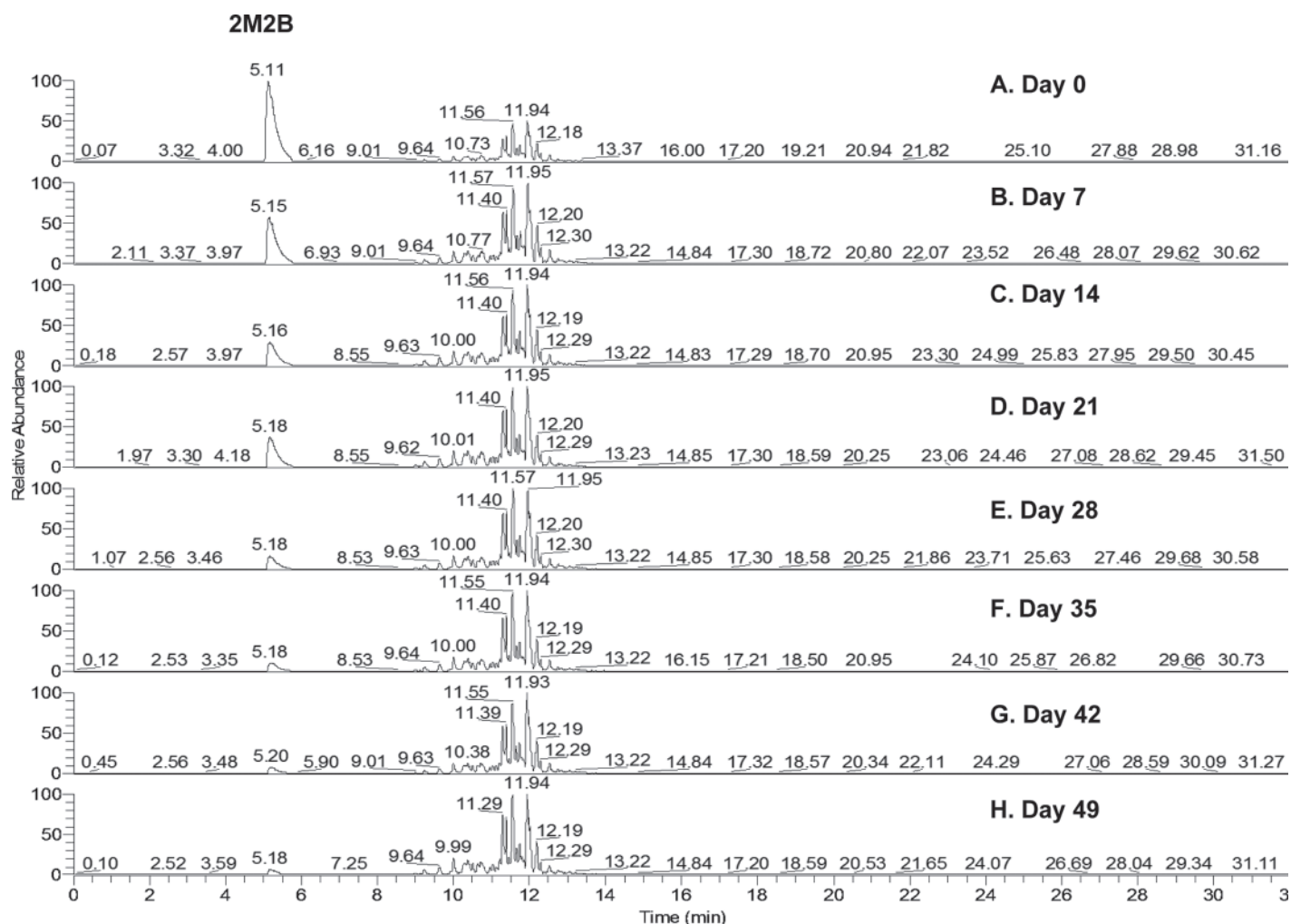
Volatile composition of plastic collars (Nurturecalm 24/7 Canine Calming Pheromone Collar, Meridian Animal Health) and liquid aerosols (Sentry Calming Spray for Dogs, Sergeant's Pet Care Products Inc.) manufactured with 2M2B (Manufacture #: 3233ADD020) was determined via SPME followed by GC-MS analysis (Trace GC Ultra coupled with ISQ single quadrupole MS; Thermo Fisher Scientific Inc., San Jose, CA). The SPME holder (Custodion, Torion Technologies Inc., American Fork, UT) was equipped with a PDMS/

DVB 65- $\mu\text{m}$  fiber (Supelco, Bellefonte, PA). A snippet of plastic collar (25 mm) or 3 mL of aerosol spray liquid was placed in a 22-mL 6 dr glass vial (Fisherbrand, Thermo Fisher Scientific Inc.) with a plastic screw top containing a custom-drilled hole for the SPME fiber. The fiber was inserted into the headspace of the glass vial for 30 min to reach equilibrium, and the fiber was removed and injected in a splitless injector of the Trace GC Ultra chromatograph heated at 220°C. Gas-chromatography analyses were conducted using ISQ Single Quadrupole GC-MS system with a 30-m, 0.25-mm i.d., 0.25- $\mu\text{m}$  d<sub>f</sub>, Rtx-5 column (Restek, Bellefonte, PA) with the following temperature program: maintained at 40°C for 3 min, heated

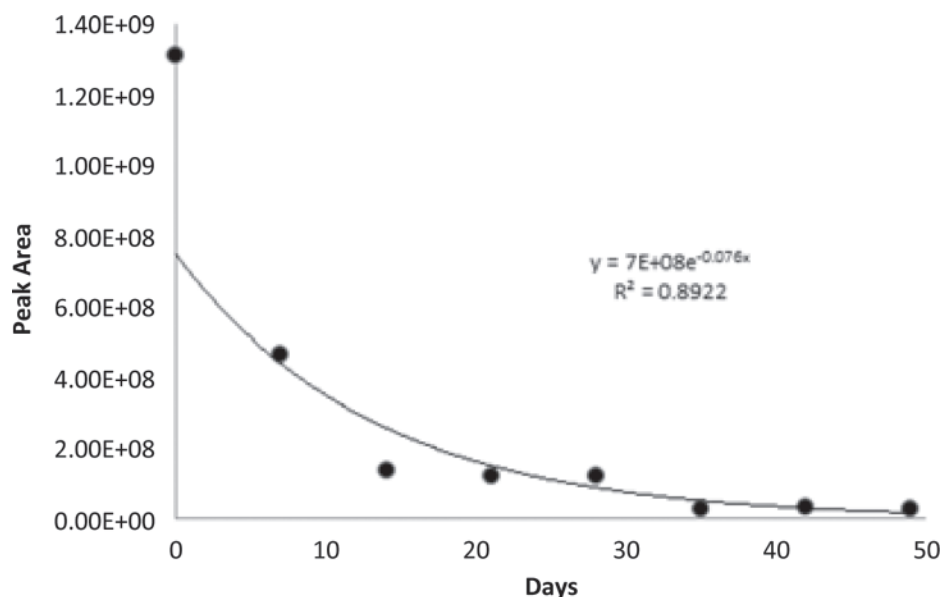
to 300°C at 10°C/min, and maintained at 300°C for 3 min. Mass spectra were recorded in electron-impact mode (70 eV) with a mass range of 40 to 450 u. To verify that 2M2B was being released by the collars, separate samples containing 2M2B diluted in isopropyl alcohol (10 g/L) were used as standards and analyzed using the same conditions as described above.

### Exp. 2: 2M2B Release over Time in the Laboratory

A new collar was removed from its package and placed in an empty beaker at room temperature (23°C) on d 0. A snippet of the plastic collar (25 mm) was analyzed via SPME followed by GC-MS using the same



**Figure 2.** The gas-chromatography traces of solid-phase-microextraction analysis for 2-methyl-2-butenal (2M2B) in the immediate air surrounding a dog collar after removal from packaging after (A) 0, (B) 7, (C) 14, (D) 21, (E) 28, (F) 35, (G) 42, and (H) 49 d. 2-Methyl-2-butenal was present at 5.11 to 5.25 min. Synthetic lavender fragrance molecules were present in the 2M2B collar at 7.24 to 14.83 min.



**Figure 3.** Relative concentration of 2-methyl-2-butenal in air surround a dog collar after removal from packaging over 49 d (7 wk) fitted to an exponential decay rate.

techniques as Exp. 1. A new piece was subsequently removed each week (d 7, 14, 21, 28, 35, 42, 49) and analyzed accordingly.

### **Exp. 3: 2M2B Residual in Collar in Behaving Dogs**

Three collars were removed from their packages and placed on 3 residential dogs of varying activity levels that the owner ranked. The first dog, Toto, was a 6-yr-old, castrated male Cairn Terrier that the owner considered most active. The second dog, Wolf, was a 7-yr-old, castrated male Pomeranian that the owner considered moderately active. The third dog, Lucky, was a 10-yr-old, spayed female Chihuahua that the owner considered least active. These collars remained on the dogs for 35 d before they were removed, and a snippet (25 mm) from each was analyzed via SPME and GC-MS using the same techniques as Exp. 1 and Exp. 2.

### **Statistical Analysis**

The relative abundance of 2M2B over time was analyzed using Curve Estimation of SPSS (V. 21; IBM Corp., Amork, NY) to determine the

best nonlinear regression model to apply. Data comparing gas chromatography traces of 2M2B in the air from the Exp. 2 collar at 35 d to the collars worn by dogs for 35 d were analyzed in Excel as a series of  $n-1$  degrees-of-freedom, 2-tailed  $t$ -tests. Effects were considered significant at  $P < 0.05$ .

## **RESULTS AND DISCUSSION**

### **Exp. 1: 2M2B Release from Collar and Liquid**

The gas-chromatography traces obtained for the standard, collar, and spray liquid displayed peaks identified with high probability as 2M2B at 5.13 to 5.17 min (Figure 1) relative to both library values and the purified sample. Comparison of the mass spectra of 2M2B in the aerosol liquid, collar, and standard to the NIST mass spectral library in GC-MS verified that the molecule was present. Isopropyl alcohol, used as the 2M2B vehicle, was verified in both the control standard and 2M2B spray at 2.30 min. Both the collar and the aerosol spray were manufactured with natural fragrances of unknown source to the investigators with an odor of lavender. These molecules account for many

of the peaks that appear throughout 7.24 to 22.81 min.

### **Exp. 2: 2M2B Release over Time in the Laboratory**

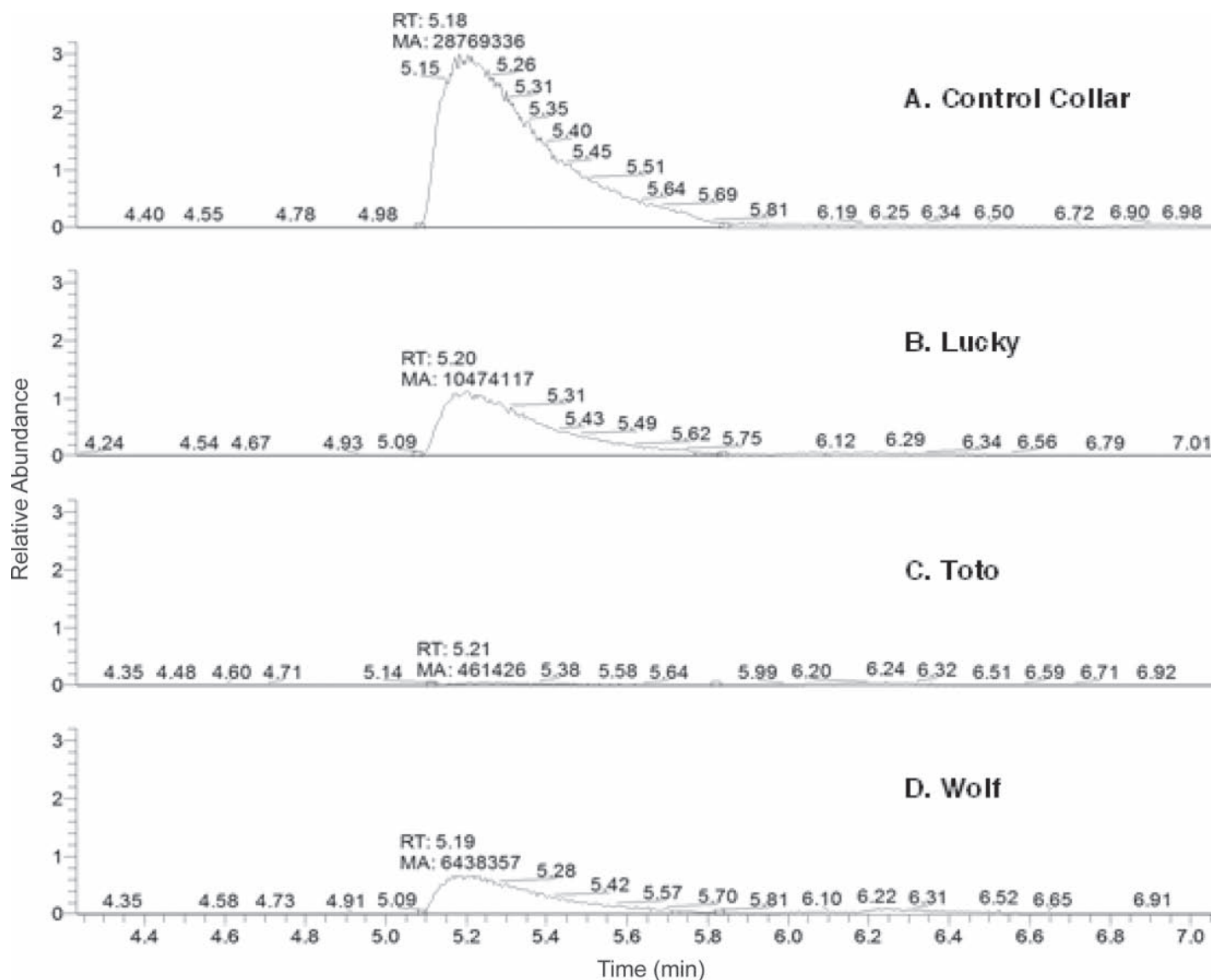
Collar 2M2B relative peak abundance at 5.11 to 5.25 min decreased over 49 d but was detectable by the GC-MS on the last day (d 49, Figure 2). Lavender fragrance molecules account for the peaks from 7.24 to 14.83 min at each of the time intervals. The GC-MS determines relative concentration by detecting the area under the curve for each peak (in units of intensity  $\times$  time), which was used to determine the rate at which 2M2B decreased. Collar 2M2B concentration decayed exponentially ( $R^2 = 0.8922$ ;  $P < 0.01$ ; Figure 3) because of diffusion.

### **Exp. 3: 2M2B Residual in Collar in Behaving Dogs**

The GC-MS results demonstrated a trend that the collars worn by all 3 dogs (Figure 4) had lower levels of 2M2B (mean = 5,791,300 units, SD = 5,037,609 units) than the room temperature equivalent (28,769,336 units) from Exp. 2 at 5.18 to 5.90 min,  $t(2) = 3.605$ ,  $P = 0.058$ . The relative amount of 2M2B was also related to the activity level, with the least amount of 2M2B present on the collar of most active dog Toto, a Cairn Terrier, followed the intermediate active dog Wolf, a Pomeranian, and the least active dog Lucky, a Chihuahua. The goal of this work was to identify sources of variation in collar delivery, not to specifically determine relationships between behavior and pheromone or interomone delivery, and further research may clarify the relationship between them.

## **IMPLICATIONS**

Head-space release of 2M2B around a commercial dog collar and calming aerosol liquid was verified via SPME and GC-MS. This is the first demonstration of pheromone or interomone



**Figure 4.** The gas-chromatography traces of solid-phase-microextraction analysis for 2-methyl-2-butenal in the immediate air around (A) the collar from Exp. 2 and collars attached to dogs (B) Lucky, (C) Toto, and (D) Wolf after 35 d. 2-Methyl-2-butenal was present at 5.18 to 5.90 min. RT = retention time; MA = manually integrated area.

release into the air around a collar or liquid by any method. Once a dog collar is removed from its protective packaging, 2M2B volatilizes at a decreasing rate at room temperature but can still be detected after at up to 49 d after opening the package. Dog-to-dog variation in collar release may be attributable to variation in dog behaviors, perhaps level of general activity. These data document that SPME can be used to determine pheromone or interomone concentrations. Second, the manufacturing process does not prevent volatilization of the pheromone or interomone 2M2B

in the collars and the spray. Finally, 2M2B can be detected for more than 30 d after the plastic collar is removed from its package.

### ACKNOWLEDGMENTS

The authors thank Texas Tech University for use of the Mass Spectrometry Facility, the Department of Chemistry and Biochemistry, and the office of the vice president for research. One author (J. J. M.) has provided consultation to Sergeant's Pet Products and Meridian Animal Health.

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