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Identification of (−)-β-Caryophyllene as a Gender-Specific Terpene Produced by the Multicolored Asian Lady Beetle

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Abstract This work reports the development and use of techniques for characterizing volatile chemicals emitted by the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), in an effort to identify the semiochemicals involved in establishment and persistence of overwintering beetle aggregations. Volatiles emitted from live beetles were detected by using whole-air sampling and solid-phase microextraction (SPME). Adsorbed volatiles were thermally desorbed and identified with gas chromatography-mass spectrometry (GC/MS). By comparing the chromatograms of volatiles emitted from live male and female beetles, a sesquiterpene, (−)-*β*-caryophyllene, was found only in the females. The identity of (−)-β-caryophyllene was confirmed by using NIST Library searches, comparing retention times with those of known standards, and by using higher-resolution GC/MS above bench top capability. Although SPME trapping detected a wider array of compounds compared to whole-air sampling, the latter method is better suited for automation. Unattended automated sampling is required for the continuous measurement of targeted compounds under dynamically changing incubation conditions. These conditions,

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mimicking natural overwintering conditions, are essential to our long-term goal of using this technology to detect and identify the aggregation pheromone of *H. axyridis*.

Keywords β-Caryophyllene · *Harmonia axyridis* · gas chromatography/mass spectroscopy (GC/MS) · terpene · solid-phase microextraction (SPME) · whole air sampling · Racemic

Introduction

The multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), is an effective predator of aphids and other soft-bodied insect pests in pecan, apple, and citrus ecosystems (Tedders and Schaefer, 1994; Brown and Miller, 1998; Michaud, 2002; Brown, 2004) during the spring and summer. Lady beetles play an important role as biological pest control agents. Nonetheless, they are considered a nuisance pest when they overwinter in North American homes. In autumn, adults leave feeding sites in agricultural and urban landscapes and search for overwintering sites. Attics, wall voids, and other inaccessible locations in houses and man-made structures are often selected (Nalepa et al., 1996; Schaefer, 2003). Odors emanating from beetles in infested locations can lead to allergic rhinoconjunctivitis (Yarbrough et al., 1999; Ray and Pence, 2004). As a defense, *H. axyridis* adults secrete hemolymph from their joints when roughly handled (King and Meinwald, 1996; Laurent et al., 2002). This secreted hemolymph can stain furniture and draperies, and gives off an unpleasant odor.

In recent years, *H. axyridis* has become a concern to grape growers and the wine industry. The inadvertent crushing of beetles with grapes releases defensive compounds (e.g., pyrazines) resulting in off-taste wines (Cudjoe et al., 2005). Production of beetle-tainted wines has been reported in several wineries in the northeastern United States and southern Canada (Pickering et al., 2004, 2005).

A potential approach to managing this useful, but problematic insect is by implementing a push–pull strategy. Such an approach relies on using environmentally friendly repellents to push beetles away from structures or grape clusters during the fall, and attractants (possibly aggregation pheromones) to pull beetles into outdoor traps (Riddick et al., 2000; Riddick and Aldrich, 2004). To begin the process of detecting and identifying volatiles that function as attractants or aggregation pheromones, research was initiated to compare airborne chemicals released from living male and female lady beetles.

Gas chromatography/mass spectrometry (GC/MS) is a common analytical technique used to identify volatile analytes and MS offers sensitivity for the detection of compounds that are in trace amounts. However, GC and GC/MS alone can not always handle sample matrices directly, and sometimes extraction and preconcentration is necessary prior to analysis. To study the chemical profile of beetle odors, we utilized two sample introduction techniques. Solid phase microextraction (SPME) was developed in the early 1990s as a means of extracting and preconcentrating pollutants in water samples (Arthur and Pawliszyn, 1990; Zhang and Pawliszyn, 1993), but has also been used for the study of airborne insect pheromones (Malosse et al., 1995). SPME involves absortion/adsorption of volatiles and semivolatiles in a sample matrix to be fused onto a chemically coated fiber. Direct desorption of the extracted compounds into the GC column occurs in the heated injection port of the GC system. In contrast, trapped whole air sampling, a modified EPA TO-14 type methodology (Environmental Protection Agency, 1989), is similar to SPME. However, a variable volume of air is removed from the sample and preconcentrated by

absorption/adsorption depending on the chemical composition of the trapping material in the trap. The trap temperature is rapidly raised to allow desorption, and a series of valves allows the carrier gas to sweep through the trap that is now in line with the GC column. Both of these techniques allow living subjects to be tested in a more natural, less-stressed environment. The solvent-free environment is unique to these techniques, and their sensitivity allows reduced sampling time over traditional methods. Both whole air and SPME extraction are time-saving procedures, which reduce stress to the insects and allows the investigation of their normal pheromone production. Traditional methods often involve solvent extractions and direct injection into a GC port via a syringe (Malosse et al., 1995; Agelopoulos and Pickett, 1998; Al Abassi et al., 1998; Robbins et al., 2003; Zhang and Aldrich, 2003; Cudjoe et al., 2005).

The primary objective of this study was to develop a useful, sensitive method for detection and identification of volatile chemicals emitted by live lady beetles.

Methods and Materials

Insect

Cultures H. axyridis adults were purchased from Rincon-Vitova Insectaries Inc. (Ventura, CA, USA) and placed at random into one of two polypropylene cages (30 × 30 × 30 cm, 24 mesh size, Bug Dorm 1™; MegaView Science Education Services Co., Ltd., Taiching, Taiwan), provisioned with food (pure honey, apple slices, lepidopteran eggs) and sterile water on cotton wads at the base of each cage. Both cages contained mixed sexes of beetles of unknown age. Cages were kept inside a plant growth chamber (at 10°C, 60–68% RH, 12-hr photophase).

Fig. 1 Environmental sampling chamber. This is a 1-l plexiglas cylindrical container, encircled with copper tubing connected to a water bath to control temperature. A small, mesh-covered, battery-operated fan is housed inside the chamber to maintain homogeneous air circulation. The chamber is equipped to accept SPME fibers and an air tube for whole air sampling

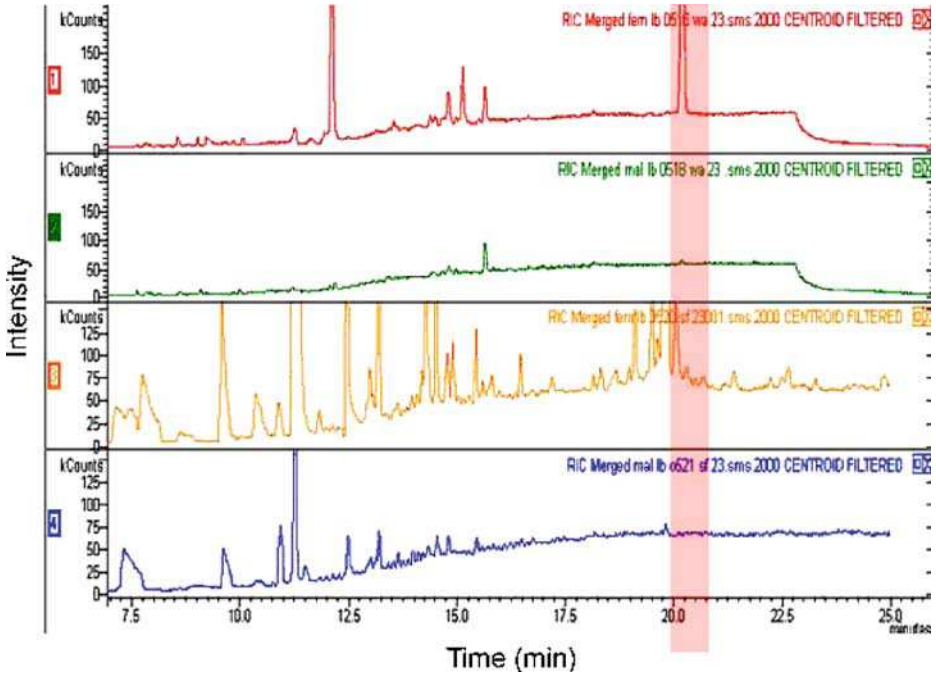

Fig. 2 Total ion chromatograms. These total ion chromatograms, obtained via GC/MS, represent air samples from with the Environmental Sampling Chamber containing 3 g lady beetles. (1) Whole air sampling of females, (2) whole air sampling of males, (3) SPME sampling of females, and (4) SPME sampling of males. Shaded peak shows an area of difference between males and females and major analyte was identified as β-caryophyllene

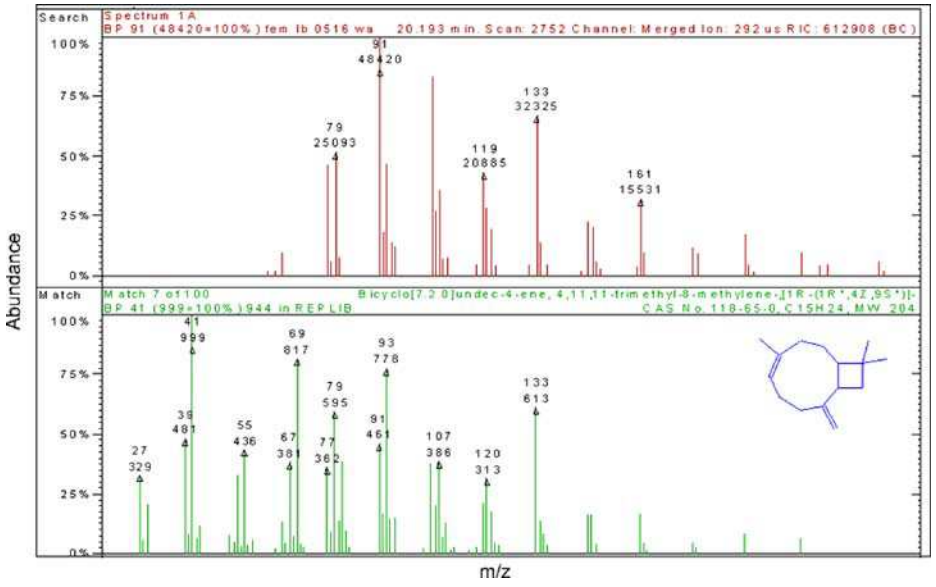

Fig. 3 Spectral match of the shaded peak to the NIST Library using data acquired on ion trap MS

Sample Preparation

H. axyridis adults were separated by gender, and approximately 3.0 g (101 males, 83 females) of insects were placed in the environmental sampling chamber for analysis. Mass was used to normalize weight discrepancy between sexes. The sampling chamber was essentially a modified, plexiglass (1 l) capillary syringe (Fig. 1). Once placed in the chamber, beetles were allowed to acclimate for 8 hr. All GC/MS sampling was performed at 24°C; temperature was kept constant throughout by circulating tap water from a water bath and through copper tubing, encircling the chamber. A small battery-operated fan was placed inside the chamber to maintain constant air circulation. A screen was placed around the fan to prevent beetles from contacting the fan blades.

A 1.0 mg/ml stock standard solution of pure β-caryophyllene dissolved in acetone was prepared and used for confirmation of analyte identification. Aliquots of these solutions were immediately sealed and kept in the refrigerator. Fresh standards were always used prior to each analysis, and all chemicals were obtained from Sigma-Aldrich.

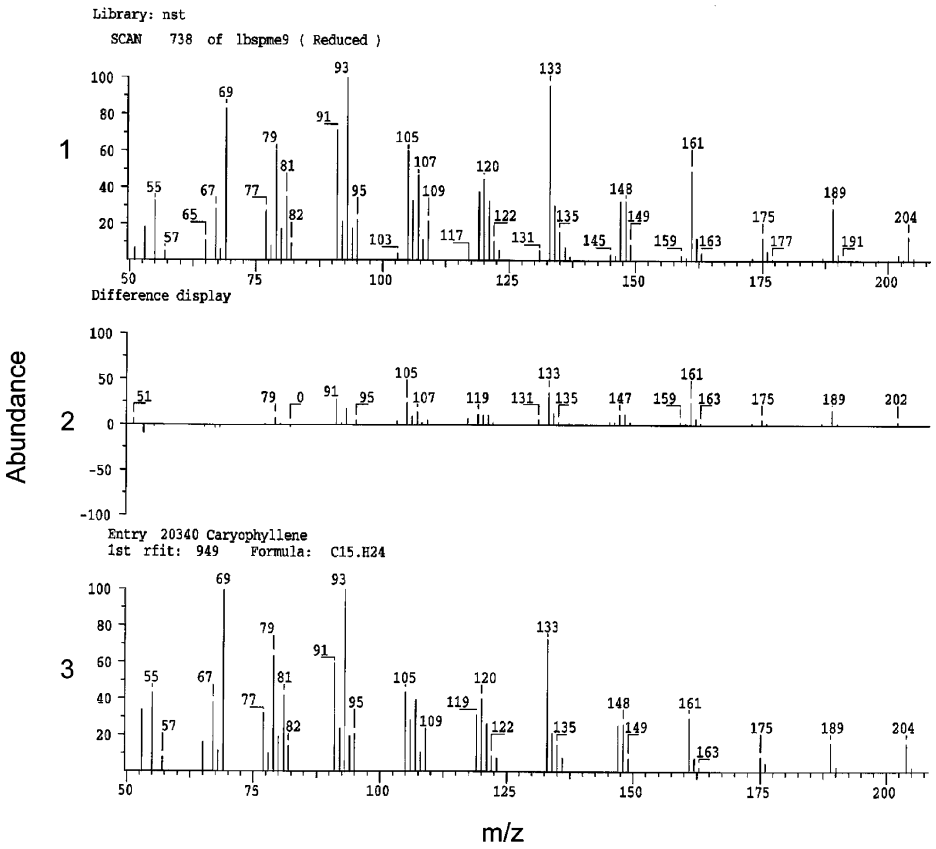

Fig. 4 Spectral identification confirmed using NIST Library and data acquired on Magnetic Sector MS. (1) Raw MS spectra of female-specific analyte corresponding to the peak represented in Fig. 2. (2) Spectral difference between the raw MS spectra and the NIST Library match. (3) NIST Library match spectra identifying and confirming the compound as caryophyllene

Sample Collection

A 75-μm carbonex/polydimethylsiloxane fiber (SPME, Supelco™), 1 cm in length, was used for volatile sequestration. Prior to extraction, the fiber was conditioned for 30 min in the injection port of the GC at 250°C. The fiber was allowed to absorb at 24°C for 2 hr and desorb for 3 min at 200°C.

A Tenax-G-Trap (Varian Chromatography Systems, Walnut Creek, CA, USA) was used for the whole air sampling. The trap was held at 4°C for 12.5 min, while 40 ml/min of sample air were collected yielding a total volume of 500 ml. Desorbtion of the trap was at 180°C for 2.5 min and then baked at 200°C for 20 min.

GC/MS Separation

A Varian Star 3600 GC with helium as a carrier gas passing through a Restek Rtx-Volatiles column (60 m × 0.32 mm, with a 1.5-μm film) coupled with the Varian Saturn 2000 GC/MS with wave board technology, which has the NIST library installed, was used for identification. Regardless of the sampling technique, the initial GC oven temperature (40°C) was held for 3 min and then ramped to 260°C at 20°C min^{-1}, and then held for another 11 min. Electron impact ionization (EI) or chemical ionization (CI) of the analytes was achieved by using the Saturn 2000, with a scan range of 60–250 *m/z* and scan time of 0.45 scan/sec. For confirmation and identification of the analytes, a Finnigan MAT 95 XL Magnetic GC/MS operating at 5000 resolution was utilized based on this instrument's extreme sensitivity and spectral resolution. The column in this magnetic sector GC/MS was a Phenomenex ZB5 (30 m × 0.25 mm, with a 0.25 μm film). Helium was used as the carrier gas, and the system was equipped with the NIST library. The higher resolution system used electron impact with a MS scan range of 50–250 *m/z* and a scan time of 1 sec/decade.

Chiral Separation

Racemic β-caryophyllene was resolved by GC on a Cyclodex-B™ capillary column (30 m × 0.25 mm i.d.; 100°C for 3 min, to 117°C at 1°/min; Advanced Separation Technologies, Inc., Whippany, NJ, USA). Since neither the plus enantiomer nor the racemic β-caryophyllene are

Table 1 Retention times (min) for caryophyllene peaks

	Trial 1	Trial 2	Trial 3	Average	SD	SEM
Whole air sampling (EI)						
Females	20.193	20.186	20.195	20.191	4.74×10^{-3}	2.74×10^{-3}
Males	NA	NA	NA	NA	NA	NA
Caryophyllene standard	20.189	20.194	20.191	20.191	2.52×10^{-3}	1.45×10^{-3}
SPME (EI)						
Females	19.879	19.816	19.742	19.812	6.86×10^{-2}	3.96×10^{-2}
Males	NA	NA	NA	NA	NA	NA
Caryophyllene standard	19.790	19.789	20.123	19.901	0.192	0.111
SPME (EI) high resolution						
Females	11.123	11.135	11.121	11.126	7.57×10^{-3}	4.37×10^{-3}
Males	NA	NA	NA	NA	NA	NA
Caryophyllene standard	11.102	11.112	11.134	11.116	1.64×10^{-3}	9.45×10^{-3}

EI: Electron impact ionization; NA: not applicable.

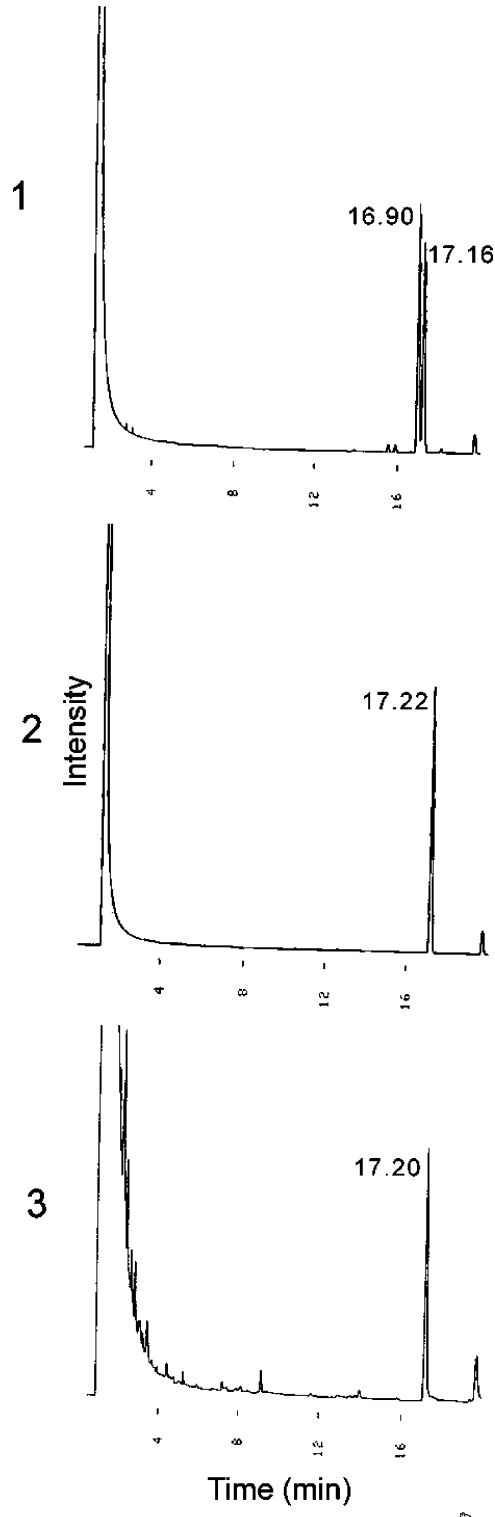

Fig. 5 Enantiomeric composition of β-caryophyllene from *Harmonia axyridis*. (1) Racemic mixture of β-caryophyllene yielding two peaks (+) 16.9 min and (−) 17.2 min, (2) pure (−)-β-caryophyllene, (3) Sample of ~500 adults

commercially available (Fricke et al., 1995), it was necessary to prepare racemic β-caryophyllene. This was accomplished by converting α-humulene [(*E*,*E*,*E*)-2,6,6,9-tetramethyl-1,4,8-cycloundecatriene; Fluka Chemical Corp., Ronkonkoma, NY, USA] to (±)-β-caryophyllene according to Greenwood et al. (1968).

Results

A comparison of volatiles emitted by male and female live beetles was achieved by using SPME and trapped whole air sampling techniques (Fig. 2). Differences in the total ion chromatograms between sexes, independent of the sampling technique, were evident. The shaded area in Fig. 2 highlights one of these areas of difference and indicates a compound that is present only in females. The identification of the analyte was obtained by a combination of comparisons with the NIST Library (Figs. 3 and 4) and by comparing the retention time with known standards (Table 1), which were analyzed by ion trap and magnetic sector GC/MS (Fig. 4). The analyte was β-caryophyllene. β-Caryophyllene (bicyclo[7.2.0]undec-4-ene 4,11,11, trimethyl-8-methylene) has a molecular weight of 204. The gaseous emissions from the same lady beetle population were analyzed three times at 2-d intervals. The average retention time run on the Ion trap MS was 20.191 ± 0.005 min for the whole air sampling and 19.812 ± 0.070 min for the SPME sampling. When pure caryophyllene was run with the same methodology, average retention times were 20.191 ± 0.003 and 19.901 ± 0.192 min, respectively, for whole air and SPME. There is greater variation in the retention times for the SPME method compared to whole air methodology. This can be attributed to the automation of the whole air method that reduces the human error and increases precision when compared to manual SPME technique.

The mass spectrum (Fig. 3) shows a fragmentation pattern with major ions of *m/z* 161, 133, 105, and 91, and gives a spectral probability of 79.3%. The match with β-caryophyllene also shows other terpene compounds that share these ions, and therefore higher-resolution GC/MS was utilized for absolute identification. The Magnetic Sector, equipped with the NIST library, confirmed β-caryophyllene as the analyte when compared with pure standard at retention time of 11.126 min. Figure 4 reveals little spectral difference between the raw analyte MS spectra and the NIST reference.

The racemic mixture of β-caryophyllene was resolved with near-baseline separation, exhibiting two peaks eluting at 16.9 and 17.2 min. (−)-β-Caryophyllene eluted at 17.2 min, establishing that the earlier eluting enantiomer was (+)-β-caryophyllene (Fig. 5). Coinjection experiments of an aeration sample of ~500 male and female *H. axyridis* adults (73 h/75 μl CH_2Cl_2) with (±)-β-caryophyllene and (−)-β-caryophyllene established that caryophyllene from *H. axyridis* consisted solely of the (−)-enantiomer with no trace of (+)-β-caryophyllene.

Discussion

This work demonstrates an innovative approach in which compounds emitted by live laboratory reared beetles, in an environmentally controlled chamber, can be analyzed with two different sampling methods. Environmental chamber design along with the development and sensitivity of the sampling techniques allows for the analysis of beetle odors without subjecting the insects to undue stress. Traditional methods have involved solvent extractions that use charcoal filters, with lady beetles packed in a small cylindrical tube for

several days (J.R. Aldrich, unpublished data). These tight quarters, in the absence of food and water, can cause unintentional die-off of some of the test beetles. The induced stress may impact the profiles of emitted volatiles. Instead of several days, our sampling time is reduced to a matter of hours, and the sample size can be reduced to ensure a more natural production of emitted volatiles.

The sampling methods removed only a small aliquot of air from the environmental chamber and did not comprehensively deplete air supply. The sensitivity of the procedure can be enhanced by withdrawing larger volumes of chamber air. This was a variable volume system in which the flow rate and/or time could be changed to facilitate the desired sensitivity of the analysis. In contrast, SPME fibers could be introduced into smaller chambers, reducing headspace, and thereby concentrating the volatiles and increasing sensitivity. This increase in sensitivity will play a greater role as we identify more peaks in the chromatography profile in order to isolate the aggregation pheromone.

The sampling methods produced similar chromatograms that showed marked differences between laboratory-reared male and female beetles. A combination of retention time, GC/MS analysis with NIST library searching, complemented by higher-resolution GC/MS allowed for the identification of one analyte. This was identified as β-caryophyllene and was exclusively emitted by the females. Preliminary data from field collected beetles exhibit similar results (A.E. Brown, unpublished data).

Isoprenoids play a major role in governing the behavior of other insects (Birkett and Pickett, 2003). For example, the boll weevil is attracted to cotton plants (Hedin et al., 1973), and the female is sexually attracted to the male by airborne volatiles (Tumlinson et al., 1969). In both cases, the attracting compounds are terpenes. β-Caryophyllene has been isolated from female boll weevils and serves as an attractant under certain conditions (Minyard et al., 1969). β-Caryophyllene serves as an attractant in other species, including the Colorado potato beetle (Khalilova et al., 1998), the carrot fly (Guerin et al., 1983), the damson hop aphid (Campbell et al., 1993), and the green lacewing (Flint et al., 1979). Plant-derived (−)-β-caryophyllene inhibits the response of the lady beetle, *Coccinella septempunctata* L., to aphid alarm pheromone [(*E*)-β-farnesene] in olfactometer bioassays (Al Abassi et al., 2000).

The detection of the (−)-enantiomer of β-caryophyllene in airborne volatiles from *H. axyridis* females was determined by chiral column GC and by comparison of the elution sequence of the racemic mixture. The minus enantiomer is commonly found in a variety of plant species (Nazaruk et al., 2002).

This study demonstrates that volatiles emitted from live laboratory-reared beetles can be analyzed by using SPME or whole air sampling coupled with GC/MS. These sampling techniques show differences in volatile and semivolatile organic chemicals emanating from *H. axyridis* males and females. Future studies might include lowering the temperature of the environmentally controlled chamber to mimic the natural conditions within overwintering aggregations. Although SPME fiber desorption appeared to be more sensitive than whole air sampling, the latter is presently more compatible with automated real-time analysis. Unattended automated analysis will allow us to study the physiological responses of *H. axyridis* to changes in temperature, light, and humidity.

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