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Determination of characteristic odorants from *Harmonia axyridis* beetles using *in vivo* solid-phase microextraction and multidimensional gas chromatography–mass spectrometry–olfactometry

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Abstract

Homeowners, small fruit growers, and wine makers are concerned with noxious compounds released by multicolored Asian ladybird beetles (*Harmonia axyridis*, Coleoptera: Coccinellidae). A new method based on headspace solid-phase microextraction (HS-SPME) coupled with multidimensional gas chromatography–mass spectrometry–olfactometry (MDGC–MS–O) system was developed for extraction, isolation and simultaneous identification of compounds responsible for the characteristic odor of live *H. axyridis*. Four methoxypyrazines (MPs) were identified in headspace volatiles of live *H. axyridis* as those responsible for the characteristic odor: 2,5-dimethyl-3-methoxypyrazine (DMMP), 2-isopropyl-3-methoxypyrazine (IPMP), 2-*sec*-butyl-3-methoxypyrazine (SBMP), and 2-isobutyl-3-methoxypyrazine (IBMP). To the best of our knowledge this is the first report of *H. axyridis* releasing DMMP and the first report of this compound being a component of the *H. axyridis* characteristic odor. Besides the MPs, 34 additional compounds were also identified. Quantification of three MPs (IPMP, SBMP and IBMP) emitted from live *H. axyridis* were performed using external calibration with HS-SPME and direct injections. A linear relationship ($R^2 > 0.9951$ for all 3 MPs) between MS response and concentration of a standard was observed over a concentration range from 0.1 ng L^{-1} to 0.05 μg L^{-1} for HS-SPME–GC–MS. The method detection limits (MDL) based on multidimensional GC–MS with narrow heart-cut approach for three MPs were estimated to be between 0.020 and 0.022 ng L^{-1}. This represents a 38.9–52.4% improvement in sensitivity compared to GC–MS with full heart-cut method. This methodology is applicable for *in vivo* determination of odor-causing chemicals associated with emissions of volatiles from insects.
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Keywords: *Harmonia axyridis*; Headspace solid-phase microextraction; Multidimensional gas chromatography–olfactometry; Odor; Methoxypyrazines

1. Introduction

The recent invasion and establishment of *Harmonia axyridis* (Coleoptera: Coccinellidae) in North America has resulted in a pest on several fronts. Ecological impacts of *H. axyridis* have been recorded in several states, including the replacement of native coccinnellids in agricultural settings [1]. Extension entomologists have received numerous complaints from urban and rural homeowners complaining of larger numbers of adult *H. axyridis* gathering in windows and attics [1]. Within the home, *H. axyridis* is a pest in several ways. The large numbers and congregating activity within the home make for a noticeable nuisance. When disturbed the defensive response of adult *H. axyridis* includes reflexive bleeding and the release of noxious compounds. These compounds include but are not limited to MPs [2–6]. MPs are very potent odorants and have a distinctive smell, similar to freshly cut green bell pepper or green peas. The human olfactory thresholds for MPs are extremely low, in the range of 2 ng L^{-1} in water [7,8].

The larvae and adults of *H. axyridis* are primarily predators and have been considered a significant source of biological control for another invasive pest, the soybean aphid, *Aphis glycines* (Hemiptera: Aphididae). However the feeding habits of *H. axyridis* in North America are more cosmopolitan, with reports of fall feeding on several fruits [2]. The impact of this feeding by adult *H. axyridis* as a significant source of yield loss is not clear. A greater threat may be a loss in fruit quality, especially grapes, when harvested fruit is contaminated with adult *H.*

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axyiridis. When processed into wine, MPs released from lady beetles have been identified as a fouling agent [9–12]. Allen and Lacey reported lower odor detection thresholds in white wine compared with red wine [9]. Pickering et al. [10] found *H. axyridis* released MPs, particularly IPMP was the agent responsible for the wine taint.

The concentration of MPs released by lady beetles (coccinellids) is in the order of pg/beetle [5] and ng L^{-1} [10–13] in wine. Therefore it is necessary to develop highly sensitive extraction and analysis methods for qualitative and quantitative purpose at such low levels. Comparison of sampling and analytical methods in previous studies to characterize odorants released by lady beetles is shown in Table 1 [2,3,10,12,14,15]. Gas chromatography has been commonly employed for this purpose due to the volatility of MPs. However, the detection of volatiles at low concentrations from complex matrixes first requires an extraction and a preconcentration step in sample preparation procedures. In addition, processing large numbers of insects is often required to determine low levels of certain volatiles. Extraction by cation-exchange resin, liquid–liquid extraction [16]; C-18 SPE extraction [10,12] and solvent extraction [2] were used to concentrate MPs from various sample matrices in previous studies. Recently, a simple one step sampling and sample preparation technique, SPME, was used for determination of MPs from wine or grape juice headspace [17–20]. SPME is solventless, reusable sampling/sample preparation technique suited for rapid qualitative and quantitative analyses [21]. It has been used to non-invasively sample aromas and fragrances emitted from plants and insects [22].

Our goal was to validate the use of headspace (HS) SPME for extraction of volatiles released by live *H. axyridis*. The same HS-SPME–MDGC–MS–olfactometry approach has been successfully used to characterize livestock odors [23–26]. This approach combines rapid sampling and sample preparation, olfactometry and multidimensional GC separation with conventional MS detector. Simultaneous isolation, identification, and analysis of volatiles and their corresponding odors improve separation and sensitivity compared to conventional, one column GC. The objective of this study was to (1) confirm if MPs are the sole source of noxious odors from *H. axyridis* using a novel approach—multidimensional GC coupled with olfactometry and (2) to determine the amounts of those characteristic odorants emitted from live *H. axyridis*.

2. Materials and methods

2.1. Standards and solutions

Reference standards used for identification were purchased from Sigma–Aldrich (St. Louis, MO, USA) and included 1,4-pentadiene (99%, purity), acetone (99%), heptane (99%), 2-butanone (99%), diacetyl (97%), 2-pentanone(99%), octane (98%), 2-methyl-3-buten-2-ol (98%), methyl benzene (99.9%), nonane (99%), isoamyl alcohol (99%), alpha-pinene (98%), camphene (95%), 3-hydroxy-2-butanone (99%), limonene (99%), octanal (99%), 6-methyl-5-hepten-2-one (99%), acetic acid (99.7%), 1,3-dichlorobenzene (98%),

Table 1
Comparison of sampling and analytical methods used to characterize VOCs emitted from insects

Ref.	Samples	Sample preparation	Chemical analyses	Odor analyses	Source and type of methoxypyrazines and other compounds of interest
This work	Live *H. axyridis* in 40 mL vial	SPME (50/30 μm DVB/Carboxen/PDMS); extraction conditions: 30 °C, 24 h SPME	MDGC–MS–O	Sniff port on MDGC–MS–O	*Harmonia axyridis*; 38 compounds; 4 characteristic odorous compounds: DMMP, IPMP, SBMP, IBMP
Cudjoe et al. [3]	Frozen and thawed *beetles* in vial	Headspace sampling, sorbent tubes and thermal desorption	GC–MS	None	*Harmonia axyridis*, *Hippodemia convergens*, *Coccinella septempunctata*; IPMP, SBMP, IBMP
Al Abassi et al. [2]	Liquid nitrogen cooled *Coccinella septempunctata*	Solvent extraction (chloroform) and evaporated. Volatiles were collected by vacuum distillation	GC–MS	GC–O	*Coccinella septempunctata*; IPMP
Pickering et al. [10]	Live *H. axyridis* were added and inoculated with juice	Samples were concentrated in a C-18 SPE cartridge and eluted by ethyl acetate	GC–MS	Sensory evaluation by panelist	*Harmonia axyridis*; IPMP, IBMP
Pickering et al. [12]	Live *H. axyridis* were added and inoculated with grape juice	Samples were concentrated in a C-18 SPE cartridge and eluted by ethyl acetate	GC–MS	Sensory evaluation by panelist	*Harmonia axyridis*; IPMP
Girsch and Foster [14]	Live and freshly dead *H. axyridis* were added to grape juice	Beetles were fermented with grape juice	None	Sensory evaluation by panelist	*Harmonia axyridis*
Zhang et al. [15]	Live *Anoplophora glabripennis* in glass container	Volatiles adsorbed by Super Q trap, and then eluted with methylene chloride	GC–MS	None	*Anoplophora glabripennis*; pheromones (dialkyl ethers, 4-(*n*-heptyloxy)butanal, 4-(*n*-heptyloxy)butan-1-ol)

nonanal (95%), 2-isopropyl-3-methoxypyrazine (97%, IPMP), 2-ethyl-1-hexanol (99.6%), propanoic acid (99%), benzaldehyde (99%), dihydro-3-methyl-2[3H]-furanone (98%), 2-*sec*-butyl-3-methoxypyrazine (99%, SBMP), 2-isobutyl-3-methoxypyrazine (97%, IBMP), isovaleric acid (99%), 1-borneol (95%), benzenemethanol (99%), phenol (99%), and indole (99%), respectively.

The three standards (IPMP, SBMP and IBMP) were used for quantification of the amount of MPs emitted from live beetles. An individual standard solution of 1 mg mL^{-1} of each MP was prepared in certified A.C.S.-grade methanol (Fisher Scientific, Pittsburgh, PA, USA). A combined standard solution containing all the analytes was prepared with each individual solution and subsequently diluted with methanol within a volumetric flask, sealed with parafilm and covered with aluminum foil to avoid photo-degradation [27]. All the solutions were stored in dark at 4 °C until use. Ultrapure-grade water from a high purity water system (Culligan Water Conditioning, Lexington, KY, USA) with conductivity 18 MΩ was used in all cases. Standard solutions used in further studies were prepared fresh by diluting different amounts of the standard solution with pure water to the required concentrations. The external calibration standard solutions ranged from 0.1 ng L^{-1} to 0.05 μg L^{-1} and were made by dilution of the stock solutions in water using optimized HS-SPME conditions. A certain volume of the standard solution were placed in 40 mL vials with a stir bar (Fisher Scientific, Pittsburgh, PA, USA) and prefilled with 20 mL of pure water and 3 g NaCl from Sigma–Aldrich (St. Louis, MO, USA). The HS-SPME extractions were preformed at 25 °C with 24 h extraction time and constant stirring. Samples were run in triplicates.

2.2. Isolation of characteristic odorants with MDGC–MS–O system

Multidimensional GC–MS-olfactometry (MDGC–MS–O) system (Microanalytics, Round Rock, TX, USA) built on a 6890N GC/5973 MS platform (Agilent Inc., Wilmington, DE, USA) were used for all analyses. This system allows for the simultaneous identification and analysis of chemicals and corresponding odors. The system was equipped with two columns in series connected by a Dean's switch. The non-polar pre-column was 12 m, 0.53 mm i.d.; film thickness, 1 μm with 5% phenyl methylpolysiloxane stationary phase (SGE BP5) and operated with constant pressure mode at 8.5 psi. The polar analytical column was a 25 m × 0.53 mm fused silica capillary column coated with poly (ethylene glycol) (WAX; SGE BP20) at a film thickness of 1 μm. The column pressure was constant at 5.8 psi. Both columns were connected in series. System automation and data acquisition software were MultiTrax^{TM} V. 6.00 and AromaTrax^{TM} V. 6.63 (Microanalytics, Round Rock, TX, USA) and ChemStation^{TM} (Agilent, Santa Clara, CA, USA). The general run parameters used were as follows: injector, 260 °C; FID, 280 °C, column, 40 °C initial, 3 min hold, 7 °C min^{-1}, 220 °C final, 10 min hold; carrier gas, GC-grade helium. The GC was operated in a constant pressure mode where the mid-point pressure, i.e., pressure between pre-column and column, was always at 5.8 psi and the heart-cut sweep pressure was 5.0 psi. The FID connected to the pre-column was maintained at 280 °C with a H_2 flow rate of 35 mL min^{-1}, an air flow rate of 350 mL min^{-1}, and the makeup N_2 flow rate of 10 mL min^{-1}. The FID data acquisition rate was 20 Hz. Mass to charge ratio (*m*/*z*) range was set between 33 and 280. Spectra were collected at 6 scans s^{-1} and electron multiplier voltage was set to 1400 V. The detection of trace three MPs was carried out using selected ion monitoring. Mass channels were *m*/*z* = 124, 137 and 152 for IPMP, *m*/*z* = 124, 138 and 151 for SBMP, *m*/*z* = 94, 124 and 151 for IBMP with 50 ms dwell times. Therefore, *m*/*z* = 137, 138 and 124 were used for quantification for IPMP, SBMP and IBMP, respectively. The MS detector was auto-tuned every day. SIM mode was used for all quantification analysis including validation of analytical method; estimation of IPMP, SBMP, and IBMP releases per beetle mass and per beetle and direct injection method.

Heart-cut valve based on Dean's switch concept was located between the pre-column and analytical column. In such a dual column system, the heart-cut valve was used to transfer specific pre-separated retention regions with characteristic *H. axyridis* odor from the pre-column (and the entire sample matrix) to the analytical column. Transferring only the selected odor-causing compounds to the analytical column was done to improve the quality of olfactometry and chemical analyses by reducing the background signals from other odorless or less relevant compounds. In this research, finding the specific odor-causing compounds was first accomplished by GC–FID–O analyses on the pre-column with trained panelists at the sniff port. The specific retention time regions with characteristic odors were selected based on reproducible odor detection start/end times. Then, in the subsequent sample analyses, the heart-cut times were set with the MultiTrax^{TM}. In the follow up GC run, only the narrow segments of chromatographic effluent that contained the characteristic odors of interest from the pre-column separation were then transferred to the analytical column and analyzed simultaneously with the MS detector and a trained panelist at the sniff port.

Sensory evaluations were made through the sniff port equipped with two capillary columns. Only one of them was allowed to deliver a sample to a panelist depending on the instrument mode. The split ratio between the MS and the sniff port was 1:3. The temperature for the sniff port capillaries was set to 220 °C to eliminate condensation. In addition, humidified air (Certified breathing air grade, 99.995% purity, Praxair, Inc., Danbury, CT, USA) was constantly delivered to the sniff port at 8.0 psi. This was done to maintain a constant humidity level for the panelists' mucous nasal membranes. The tip of the sniff port was equipped with a glass nose cone (SGE, Austin, TX, USA). The olfactory responses of panelists were recorded as aromagrams using Aromatrax software by applying an odor tag (odor event) to a peak or a region of the chromatographic separation. The odor tag (odor event) consisted of editable odor character descriptors, an odor event time span and perceived odor intensity. The aroma intensity was measured with a 0–100% point scale (100% indicated strongest odor and 0% indicated no odor). Panelists with extensive GC–O experience were trained on standard methoxypyrazines and practiced extensively with live beetle samples before controlled experiments. Three trained

panelists analyzed headspace volatiles from live *H. axyridis*. Panelist responses were compared based on odor character and odor intensity associated with separated compounds.

2.3. Data analysis

Three sets of signals were generated for each sample including the total ion chromatogram (TIC), the FID signal, and the Aromagram. The TIC was generated from gases extracted from headspace samples by the MSD in HC mode (GC–MS–O) including full HC (the heart-cut valve was open between 0.05 and 35 min run-time) and HC (the heart-cut valve was open on specific time range of interest based on GC-FID-O mode). Each TIC consisted of mass spectra for each compound and were combined and plotted as a function of retention time. Chromatogram for the FID contained a non-zero signal when the instrument was operated in no HC mode (GC-FID-O). For the no HC mode, the gas sample passed only through the non-polar precolumn and entered the FID without going into the polar column. Compounds were separated in the precolumn and the chromatogram of the sample was plotted in the FID graph. Based on this ‘screening’ in GC-FID-O mode, the heart-cut range was determined by recognizing what are needed further separation in multidimensional GC–MS–O mode.

Compounds were identified with three sets of criteria: (1) matching of the retention time on the MDGC capillary column with the retention time of pure compounds run as standards, (2) matching mass spectrums (>70%) of unknown compounds with BenchTop/PBM (Palisade Mass Spectrometry, Ithaca, NY, USA) MS library search system and spectrums of pure compounds, and (3) matching odor character.

2.4. In vivo headspace SPME of compounds released by live H. axyridis

H. axyridis were collected as needed in Ames, Iowa in September 2005, February and August 2006. *H. axyridis* were maintained in a 500 mL ventilated plastic bottle containing cotton swabs with water at room temperature (∼20 °C) prior to experiments. Field collected beetles were separated by color, into two groups of either yellow or orange. Multiple sets of randomly-selected five live from each group of *H. axyridis* were then placed in screw-capped vials (40 mL, Supelco, Bellefonte, PA, USA) sealed with a polytetrafluoroethylene (PTFE)-lined silicone septum and used for *in vivo* HS-SPME. The vials were cleaned with powered detergent (Alconox, Inc., NY, USA) and were rinsed with hot water and pure water, then dried at 110 °C overnight prior to use. Empty vials and with five live *H. axyridis* within each vial were weighed by an electronic balance. Each vial with beetles was allowed to equilibrate for 24 h before HS-SPME at 30 °C. Headspace SPME sampling was initiated by piercing the septum with the SPME needle and exposing the SPME fiber to the gases in the vial. Sampling of headspace volatiles with SPME were performed with a manual fiber holder from Supelco (Bellefonte, PA, USA). Headspace samples from live beetles only were considered for analyses, i.e., if the beetles died during sampling, the samples were discarded.

Five types of SPME fibers were first examined for MPs extraction efficiency: polydimethylsiloxane (PDMS) 100 μm, 85 μm polyacrylate (PA), 50/30 μm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS), 65 μm divinylbenzene/polydimethylsiloxane (DVB/PDMS), and 85 μm Carboxen/PDMS (Supelco, Bellefonte, PA). New fibers were used in this study. We counted the extraction–desorption times for each fiber to be less than 40 times maximum for the most used SPME fibers in this study. We did not observe any detrimental effects related to SPME fiber longevity nor we observed any problems with the reproducibility. We routinely check the quality of new (unused fibers) under microscope and return them to manufacturer if there are obvious cracks and flaking that will affect the performance of a SPME fiber. It is critically important to check the performance of the SPME fiber coatings especially when they are being heavily used and perform extractions from liquid phase.

Before use, each fiber was conditioned in a heated GC splitless injection port under helium flow according to the manufacturer’s instructions. Sampling time for HS-SPME of MP standards was first varied from 1 min to 24 h at 30 °C to determine the optimal SPME extraction conditions ensuring detection of characteristic odorants. After extraction, the SPME fiber was removed from the vial and immediately inserted into the injection port of GC for analysis. The desorption time of SPME fiber was 40 min at 260 °C. The desorption time was not optimized since it had no effect on the overall throughput of samples analyzed with a fulltime panelist participation.

2.5. Direct injection method procedure

An individual standard solution of 1 mg mL^{-1} of each MP was prepared in certified A.C.S.-grade methanol (Fisher Scientific, Pittsburgh, PA) and stored in dark at 4 °C. Stock standard solutions containing all analytes were prepared and subsequently diluted with methanol for six different concentrations (10, 50, 100, 500, 1000, and 10,000 μg L^{-1}). For direct injections, 1 μL of three MPs standard solution was directly injected into GC–MS under splitless mode condition by using 10 μL gastight syringe (Hamilton, Reno, NV, USA).

2.6. Repeatability, reproducibility, and method detection limits

The repeatability was calculated at different levels of concentration: 0.1, 0.5, 5, and 10 ng L^{-1}, and 0.05 μg L^{-1} ($n = 3$). Data were analyzed and compared using means and relative standard deviation (RSD). The reproducibility was evaluated for 0.1 ng L^{-1} ($n = 10$) by conducting repeated analyses in three different days. The U.S. Environmental Protection Agency (USEPA) methodology for estimation of method detection limits (MDLs) was used [28]. The MDLs were defined as the minimum concentration of a substance that can be measured and reported with 99% confidence when the analyte concentration is greater than zero and is determined from analysis of a sample in a given matrix containing the analyte [28]. The MDLs for MPs was

estimated using Eq. (1):

$$MDL = s \times t_{(n-1,\, 1-\alpha=0.99)} \quad (1)$$

where n is the number of replicate spike determinations at 1–5 times the estimated MDL, s the standard deviation of measured concentrations of n spike determinations, and t is the Student's t value at $n-1$ degree of freedom and $1-\alpha$ (99%) confidence level. When $n=10$ and $\alpha=0.01$, then $t=2.821$, and α = level of significance.

3. Results and discussion

3.1. Selection of SPME fiber coating

To complete our objective of confirming if MPs are the sole source of noxious odors from *H. axyridis*, we determined which SPME fibers were most sensitive for extracting target analytes (Fig. 1). The 50/30 μm DVB/Carboxen/PDMS fiber was the most efficient in extracting target MPs from headspace. Thus, the 50/30 μm DVB/Carboxen/PDMS fiber coating was selected for the subsequent experiments. This finding was consistent with Hartmann et al. [17] and Chapman et al. [29] who used DVB/Carboxen/PDMS for extracting MPs from wine.

3.2. Effects of SPME extraction time on MPs and characteristic odors released from live H. axyridis

To determine the relationship between time and MPs extraction we varied exposure periods of SPME fibers to the headspace of five live *H. axyridis* from 1 min to 24 h at a constant temperature (30 °C). Mass selective detector response measured as the mean peak area counts for these four MPs and their single ion integration were used to evaluate the effects of SPME extraction time on those characteristic compounds released by live *H. axyridis* (Fig. 2). Abundance of MPs followed a linear trend with increasing SPME extraction time for all compounds tested,

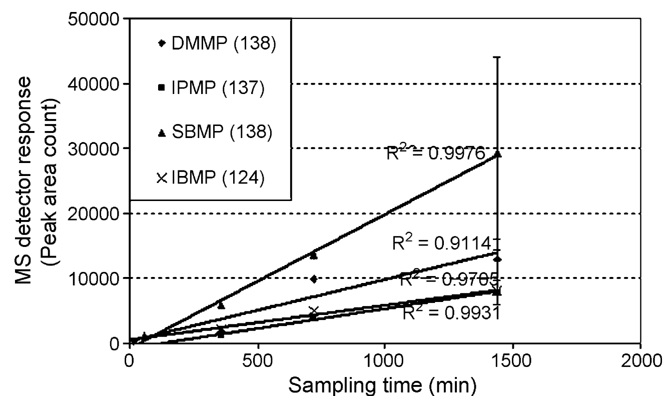

Fig. 2. Effect of HS-SPME extraction time for four methoxypyrazines released by live *H. axyridis* with 50/30 μm DVB/Carboxen/PDMS fiber. Extraction time = 1 min, 15 min, 1 h, 6 h, 12 h and 24 h. Error bars show the standard deviation of the mean (n = 3). Number in parentheses is the single ion of each compound used for peak area count integration. MS scan mode: total ion scan.

especially SBMP and IPMP ($R^2 > 0.99$). Adsorbed amounts of MPs progressively increased with no evidence of reaching sorption equilibrium within the test time range (up to 24 h). There was also an effect of SPME extraction time on odor intensity for the series of MPs tested (Fig. 3). In general, longer extraction time resulted in an increase in the odor intensity associated with these characteristic compounds. In all subsequent experiments, extraction time was set at 24 h.

3.3. Simultaneous chemical and sensory identification of VOCs released from live H. axyridis

Insect volatiles were analyzed on a multidimensional GC–MS–O system enabling simultaneous identification and analysis of chemicals and corresponding odors. Comparison of a typical total ion chromatogram and aromagram is shown in Fig. 4. Thirty-eight compounds belonging to 10 chemical groups were identified from the headspace of samples of five live *H. axyridis*, i.e., alkanes and alkenes (4), alcohols (8), aldehydes (3), aromatic hydrocarbons (1), acids (3), halogenated hydrocarbons (1), ketones (9), pyrazines and N-

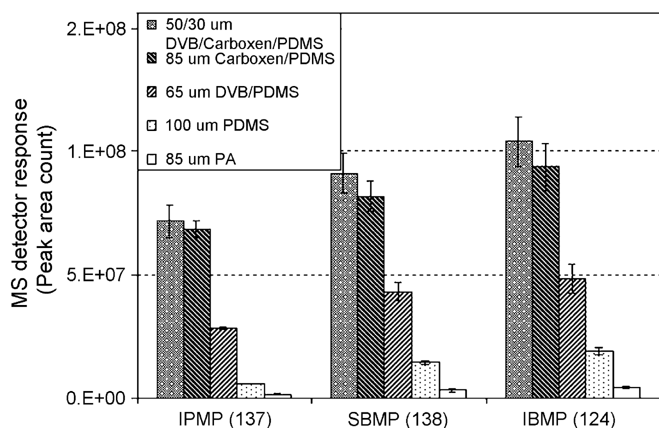

Fig. 1. Comparison of extraction efficiency of 1 ppm IPMP, SBMP, IBMP from standard solution for the 85 μm Carboxen/PDMS, 50/30 μm DVB/Carboxen/PDMS, 65 μm DVB/PDMS, 85 μm PA and 100 μm PDMS SPME fibers. Extraction conditions: extraction temperature = 30 °C, extraction time = 1 h. Number in parentheses is the single ion of each compound used for peak area count integration. MS scan mode: total ion scan. Error bars show the standard deviation of the mean (n = 3).

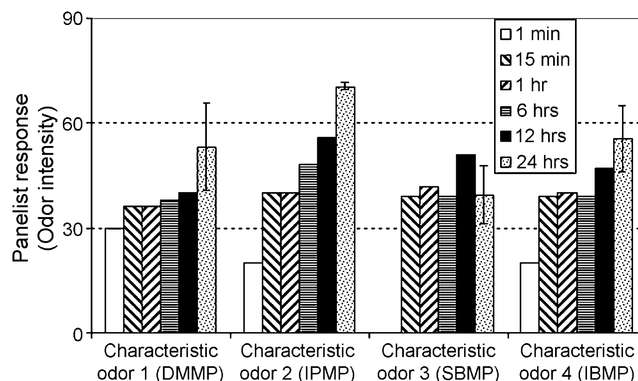

Fig. 3. Effect of time on HS-SPME extraction of the odor intensity of four characteristic odors released by live *H. axyridis* with 50/30 μm DVB/Carboxen/PDMS fiber. Extraction time = 1 min, 15 min, 1 h, 6 h, 12 h and 24 h. Error bars show the standard deviation of the mean (n = 3).

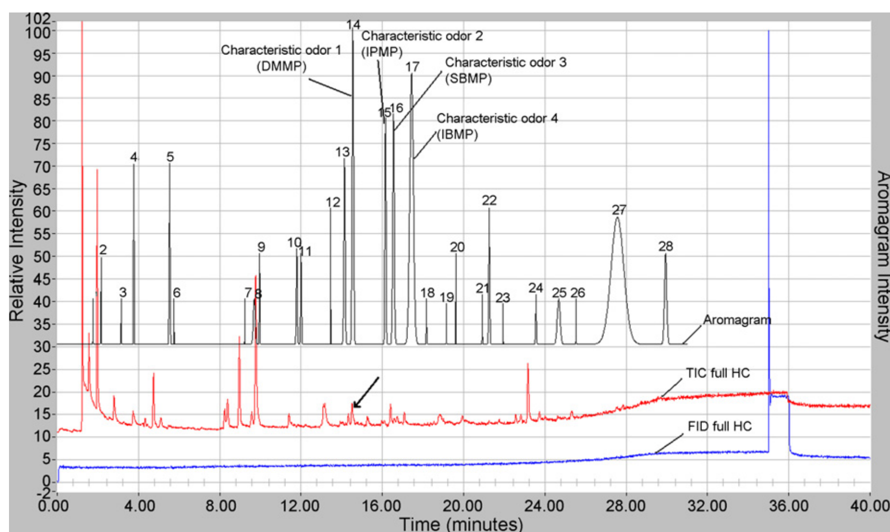

Fig. 4. Comparison of total ion chromatogram and aromagram with full heart-cut mode of headspace gases released by live *H. axyridis* in September (2005) and collected with 50/30 μm DVB/Carboxen/PDMS SPME using 24 h sampling time. Identified compounds, odors and aromas are summarized in Table 2. DMMP = 2,5-dimethyl-3-methoxypyrazine; IPMP = 2-isopropyl-3-methoxypyrazine; SBMP = 2-*sec*-butyl-3-methoxypyrazine; IBMP = 2-isobutyl-3-methoxypyrazine. Arrow marks odorous 2-ethyl-1-hexanol co-eluting with IPMP in this GC–MS–O mode.

containing compounds (5), S-containing compounds (1) and terpenes (3). A summary of detected compounds and the corresponding odor characters recorded by panelists is provided in Table 2 [30,31]. Thirty-two of these compounds were confirmed with pure standards. However, we were unable to locate commercial sources for the remaining six compounds, i.e., 2-ethyl-5-methylthiopene (CAS: 40323-88-4), 3,4-dimethyl-2-hexanol (CAS: 19550-05-1), 2,5-dimethyl-3-methoxypyrazine (CAS: 19846-22-1), dihydro-4-methyl-2[3H]-furanone (CAS: 1679-49-8), 5-ethyldihydro-5- methyl-2[3H]-furanone (CAS: 2865-82-9) and ionol (CAS: 4130-42-1). Therefore their identification is based on high probability matches (>70%) with BenchTop/PBM mass spectral library. An additional eight unidentified compounds with their aroma tags are listed in Table 2, i.e., 'sewer, skunky, fecal' (aroma peak #1, start time = 1.75 min), 'foul' (aroma peak #3, start time = 3.13 min), 'sweet' (aroma peak #5, start time = 5.48 min), 'mushroom, earthy, moldy (aroma peak #11, start time = 11.95 min), 'burnt' (aroma peak #18, start time = 18.14 min), 'earthy, moldy' (aroma peak #20, start time = 21.20 min), 'moldy, musty' (aroma peak #22, start time = 21.20 min), and 'herbaceous' (aroma peak #23, start time = 21.94 min). These compounds were extracted below the GC–MS detection limit but higher than its odor detection threshold, i.e., their presence was detected by panelists through GC–O and not readily apparent on the resulting total ion chromatogram. This illustrates cases where the use of human nose as a detector in analytical work could be advantageous in finding and identifying of compounds that are 'overlooked' and 'missed' even with sensitive chemical detectors.

3.4. Identification of methoxypyrazines released by live H. axyridis

According to previous studies, it is well known that pyrazines are secreted by lady beetles [2–5]. In this study, the four characteristic odors closely resembling the entire headspace of live beetles were identified as DMMP, IPMP, SBMP and IBMP (Table 2). The familiarity with the overall odor of beetles and their 'characteristic' odor was determined in preliminary training of all panelists. We kept live beetles in 40 mL vial for several hours and then opened the cap of the vial, and panelists smelled the odor from the opened vial as part of the training. This organoleptic evaluation of the headspace was continued during the controlled experiments. The only difference was that these evaluations were conducted after SPME extractions from the headspace. The overall odor of live beetles was a mix of 'roasted peanut, nutty, potato, green bell pepper' odor characters.

In order to identify the characteristic odors from live *H. axyridis*, three panelists analyzed headspace volatiles released by live *H. axyridis* through a sniff port. The panelists consistently identified four 'characteristic' odors, describing them as either 'moldy', earthy', 'green bell pepper', 'potato', 'peanut', 'nutty' that resulted from four MPs emitted from the headspace of live *H. axyridis*. The average odor intensity of four MPs for three panelists was 58% for DMMP, 71% for IPMP, 36% for SBMP and 59% for IBMP, respectively. The odor intensity of IPMP was the highest among all MPs identified. The reproducibility of the odor intensity of three panelists expressed as RSD were 19% for DMMP, 1% for IPMP, 15% for SBMP and 17% for IBMP, respectively. Besides these four MP's, there were several other compounds with 'nutty', 'moldy' and/or 'musty' descriptors identified by panelists as well, including: 'camphene' (compound #14), 'mushroom/earthy/moldy' (aroma peak #11), 'earthy/moldy' (aroma peak #20), 'moldy/musty' (aroma peak #30) and 'musty' (aroma peak #27). However, the average odor intensity of these compounds emitted from headspace of live *H. axyridis* was less than 20% and the odor duration time of these compounds was very short. Therefore we do not consider these compounds as a significant source of the characteristic odor of live *H. axyridis*.

Table 2
Identification of VOCs released from live *H. axiridis*

Compound #	Aromagram peak #	Retention time (min)	Compound	CAS	MW	Odor threshold (ppb) [30]	Odor character	Flavornet [31]
1		1.51	1,4-Pentadiene[a]	591-93-5	68.12			
	1	1.75					Sewer, skunky, fecal	
2	2	1.93	Acetone[a]	67-64-1	58.08	14,454	Ketone	
3		2.53	Heptane[a]	142-82-5	100.21	9,772		Alkane
4		2.75	2-Butanone[a]	78-93-3	72.11	7,762		Ether
	3	3.13					Foul	
5	4	3.68	Diacetyl[a]	431-03-8	86.09	4.4	Buttery	
6		4.16	2-Pentanone[a]	107-87-9	86.14	1,548		
7		4.41	Octane[a]	111-65-9	114.23	5,754		Alkane
8		4.66	2-Methyl-3-buten-2-ol[a]	115-18-4	86.14			
	5	5.48					Sweet, flora	
9	6	5.68	Methyl-benzene[a]	108-88-3	92.14	1,549	Sweet	Paint
10		5.86	2-Ethyl-5-methylthiopene	40323-88-4	126.05			
11		7.05	Nonane[a]	111-84-2	128.26	1,259		Alkane
12		8.18	Isoamyl alcohol[a]	123-51-3	88.15	44.7		Whiskey, malt, burnt
13		8.3	Alpha-Pinene[a]	80-56-8	136.24	692		Pine, turpentine
14	7	8.86	Camphene[a]	79-92-5	136.24		Peanut	Camphor
15	8	9.7	3-Hydroxy- 2-butanone[a]	513-86-0	88.11		Buttery, nut	Butter, cream
16	9	9.75	3,4-Dimethyl-2-hexanol	19550-05-1	130.23		Sweet, flora	
17	10	11.36	Limonene[a]	138-86-3	136.24	437	Milky, citrus	Lemon, orange
18		11.78	Octanal[a]	124-13-0	128.22	1.35		Fat, soap, lemon, green
	11	11.95					Mushroom, moldy	
19		12.16	6-Methyl-5-hepten-2-one[a]	110-93-0	126.2	38		
20	12	13.05	Acetic acid[a]	64-19-7	60.05	144	Acidic, sour	
21		13.9	1,3-Dichloro-benzene[a]	541-73-1	147			
22	13	14.03	2,5-Dimethyl-3-methoxypyrazine	19846-22-1	138.08	0.002	Characteristic, Moldy, earthy	
23		14.16	Nonanal[a]	124-19-6	142.24	2.24		Fat, citrus, green
24	14	14.43	2-Isopropyl-3-methoxypyrazine[a]	25773-40-4	152.2	0.002	Characteristic, peanut, potato	Peas, earth
25		14.45	2-Ethyl-1-hexanol[a]	104-76-7	130.23	245		
26		14.68	Propanoic acid[a]	79-09-4	74.08	35.5	Fatty acid	
27		14.9	Benzaldehyde[a]	100-52-7	106.13	417		Almond, burnt sugar
28		15.93	Dihydro-3-methyl-2[3H]-furanone[a]	1679-47-6	100.05			
29	15	16.05	2-*sec*-Butyl-3-methoxypyrazine[a]	24168-70-5	166.11	0.002	Characteristic, nutty, potato, peanut	
30	16	16.35	2-Isobutyl-3-methoxypyrazine[a]	24683-00-9	166.22	0.002	Characteristic, peanut, potato	Earth, spice, green pepper
31		16.37	Dihydro-4-methyl-2[3H]-furanone	1679-49-8	100.05			
32	17	17.01	Isovaleric acid[a]	503-74-2	102.13	2.45	Body odor, fatty acid	Sweat, acid, rancid
33		18.2	5-Ethyldihydro-5-methyl-2[3H]-furanone	2865-82-9	128.08			
	18	18.14					Burnt	
34	19	18.33	1-Borneol[a]	464-45-9	154.3	2.09	Burnt, plastic	Camphor
	20	19.60					Earthy, moldy	
35	21	20.56	Benzenemethanol[a]	100-51-6	108.14		Sweet, flora	
	22	21.20					Moldy, musty	
	23	21.94					Herbaceous	
36	24	22.5	Phenol[a]	108-95-2	94.11	110	Phenolic, medicinal	Phenol
37	25	23.66	Ionol	4130-42-1	234.39		Phenolic	
	26	25.52					Solvent	
	27	26.81					Musty, moldy	
38	28	28.65	Indole[a]	120-72-9	117.15	0.032	Barnyard,	Mothball, burnt

The compound and aroma peak number refers to Fig. 4. Odor character refers to the descriptors used by panelists in this study. Flavornet database summarizes odor descriptors.

[a] Confirmed with pure standard.

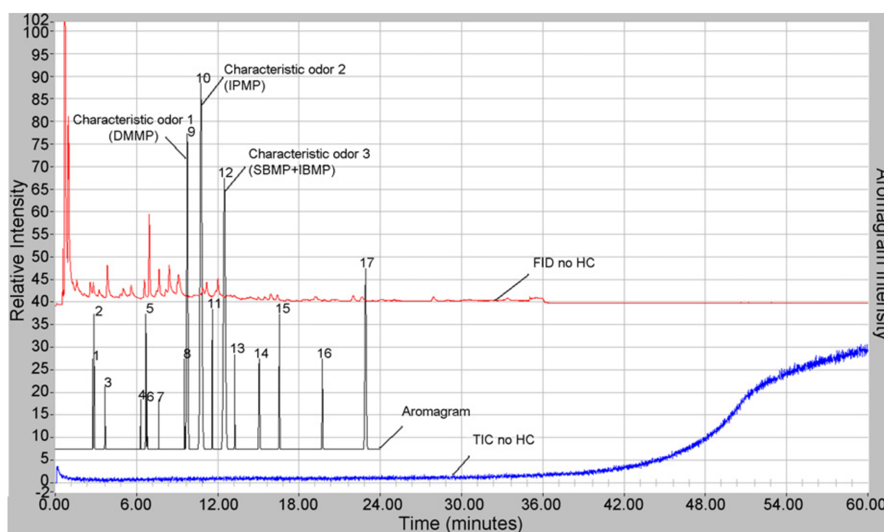

Fig. 5. Separations in GC-FID-O mode with no heart-cut: comparison of chromatogram (FID) and aromagram for the headspace of five live *H. axyridis* and collected with 50/30 μm DVB/Carboxen/PDMS SPME using 24 h sampling time, extraction temperature = 30 °C.

SBMP was positively identified by matching the retention time of standard compound, matching mass spectrum of unknown compound with BenchTop/PBM and by matching the odor character, i.e., 'bell pepper', 'peanut', and 'potato'. The extracted amount of IBMP from *H. axyridis* was lower than its identification limit (0.022 ng L^{-1}, later discussed in Table 4) based on probability matching. However, IBMP was identified by matching the retention time of standard compound and matching the odor character. One compound was consistently tagged by all panelists with the characteristic odor, i.e., 'roasted peanuts' and later tentatively identified as DMMP with a mass spectrum match greater than 90% when using the BenchTop/PBM library. Seifert et al. [8] reported 'roasted peanut' aroma and tentatively associated it with methyl MPs without specifically pointing to DMMP. The release of IPMP, SBMP, and IBMP from dead beetles has been reported in previous studies (Table 1) [2,3]. However, we are not aware of any previous report of DMMP released by *H. axyridis*. Because pure DMMP is not commercially available, it could not be confirmed with a standard at this time. However, based on this preliminary chemical and sensory identification, it is important to consider DMMP as another important, fouling odor compounds that is emitted by live *H. axiridis*.

Previous studies suggested that IPMP is the most important component of *H. axyridis*'s aroma. Cudjoe et al. [3] found IPMP was the most abundant MPs released by dead *Coccinella septempunctata*, *H. axyridis* and *Hippodemia convergens* lady beetles (Coccinellidae). Pickering et al. [10] reported IPMP was detected at relatively high concentration and at levels above sensory threshold in grape juice used for wine fermentation and contaminated with live *H. axyridis*. Pickering et al. [10] also found that IPMP is responsible for the distinctive sensory characteristics of *H. axyridis* contaminated wines and found significant positive correlations between IPMP concentration and specific aroma attributes in wines. Al Abassi et al. [2] tentatively identified IPMP from dead *C. septempunctata*, and confirmed IPMP is responsible for the characteristic odor of *C. septempunctata* adults (Table 1). The identification was confirmed by peak enhancement on GC with pure standard and by the panelist matching the aroma characteristics. In summary, only tentative identifications of IPMP were achieved using selected ion monitoring of volatiles and comparison of GC retention time in previous studies [2,3,6,10].

3.5. Multidimensional GC–MS–O

Odor and chemical separation of IPMP and other MPs from a complex matrix of insect volatiles can be challenging even with extended GC runs and other chromatographic tools. This makes it difficult to evaluate their odor impacts when analyzing the entire sample in a GC–MS–O mode. For example, IPMP coeluted with 2-ethyl-1-hexanol marked by arrow in Fig. 4. The odor character of 2-ethyl-1-hexanol is 'rose' and 'green' [17] which may be confused with IPMP. Thus, multidimensional GC–MS–O was used to (a) improve the isolation and separation of IPMP and other MPs from interferences, (b) to improve identification in the complex matrix, and (c) to separate and evaluate their odor impact. The dual-column GC system equipped a 'heart-cut' valve can divert (and isolate) a specific retention region with compounds and aroma of interest from the pre-column (non-polar) to the analytical column (polar) to enhance resolution and to minimize the interferences from coeluting compounds and aromas.

The instrument was first set to GC-FID-O mode with no heart-cut by utilizing the sniff port to identify specific GC pre-column retention times for which eluants exhibit characteristic odor. Fig. 5 shows comparison of total ion chromatogram (TIC), FID chromatogram and aromagram of volatiles released by five live *H. axyridis* analyzed in GC-FID-O mode. Based on samples analyzed in GC-FID-O mode, the specific GC pre-column retention times associated with characteristic odors were then selected for activating the multidimensional GC–MS–O mode with the Dean's switch. At first, only three characteristic odors were identified by panelists in the GC-FID-O mode. Due to

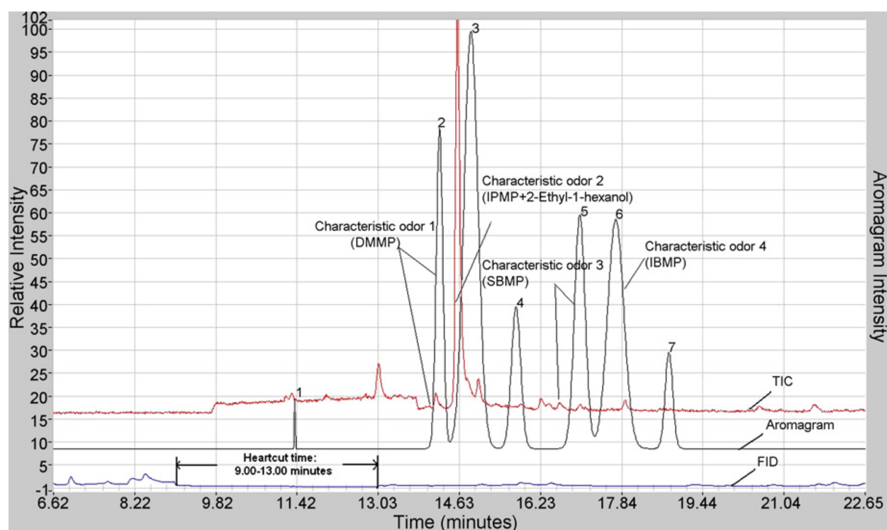

Fig. 6. Separations for MPs from the headspace of five live *H. axyridis* in MDGC–MS–O mode with heart-cut between pre-column and analytical column: comparison of the FID chromatogram, total ion chromatogram and aromagram isolating only characteristic odorants. Heart-cut time range: 9.00–13.00 min.

limited separation capacity of pre-column resulting in two of the MPs coeluting, i.e., SBMP and IBMP, the odor events were merged. When the pre-column heart-cut times were set from 9.00 to 13.00 min and a second replicate was analyzed, only heart-cuts (small segments) of chromatographic effluent were further separated on analytical column and analyzed simultaneously by the MS detector and a panelist at the sniff port. Resulting total ion chromatogram, FID chromatogram and aromagram of heart-cut effluent in MDGC–MS–O mode of volatiles released by *H. axyridis* is shown in Fig. 6. The number of aromas was significantly reduced to 7 from 28 in GC–MS–O mode (Fig. 4). However, IPMP still coeluted with 2-ethyl-1-hexanol. Then, narrower heart-cut times were set from 9.00 to 11.50 min to try to isolate IPMP from 2-ethyl-1-hexanol. As can be seen in Fig. 7, the separation of IPMP and 2-ethyl-1-hexanol was much improved even though it was not a baseline separation. Furthermore, the number of aromas was reduced to 2 making it easier for the panelist to record aroma events and then to establish the link with specific chemical. The EI-MS spectrum of IPMP from live *H. axyridis* is shown in Fig. 8 (Part A) which was almost identical to that for the standard of IPMP. The match of MS spectrum by BenchTop/PBM was 95%. The MS spectrum for the pure standard IPMP was provided in Fig. 8 (Part B) for comparison.

The odor character of IPMP, SBMP and IBMP was very similar to that of ‘green bell peppers’ and ‘potatoes’. The odor character of DMMP has been described as ‘moldy’ and ‘earthy’ (Table 2) and was consistent to the ‘roasted peanut’ and ‘moldy’ character reported by Seifert et al. [8]. The odor detection threshold of these MPs in air is not known but likely extremely

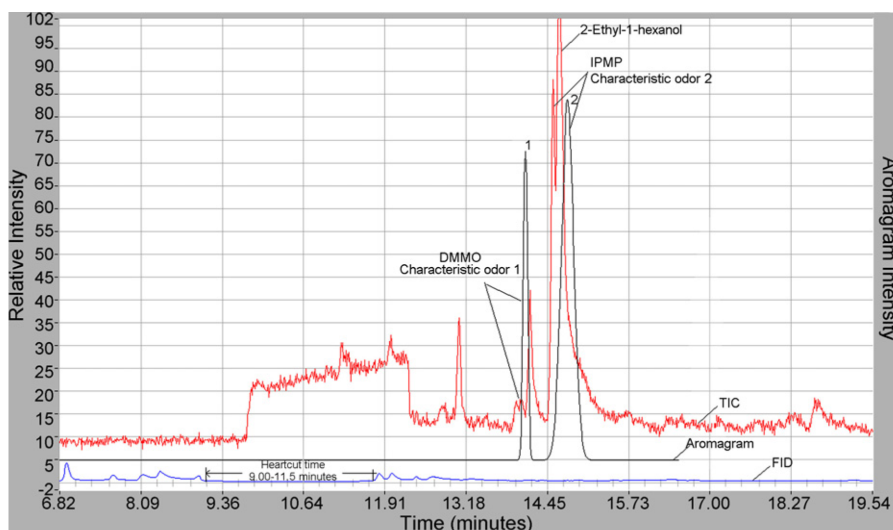

Fig. 7. Separations for MPs from the headspace of 5 live *H. axyridis* in MDGC–MS–O mode with heart-cut between pre-column and analytical column: comparison of the FID chromatogram, total ion chromatogram and aromagram isolating only characteristic odorants. Narrower heart-cut time range: 9.00–11.50 min was used to isolate aromas caused by IPMP and 2-ethyl-1-hexanol.

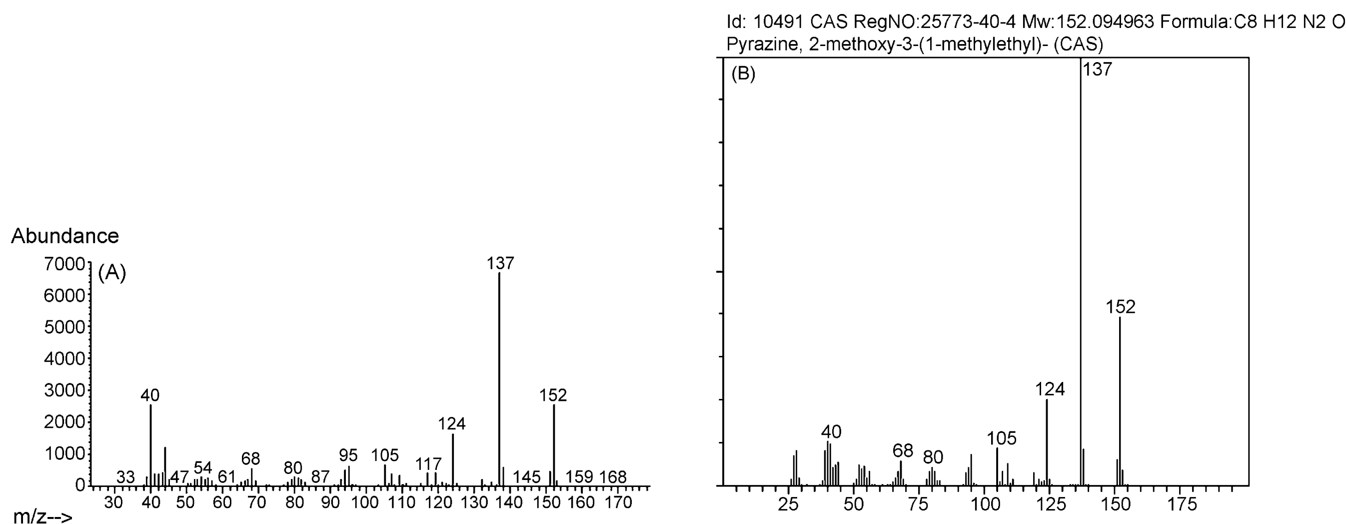

Fig. 8. The electron impact-mass spectrum (EI-MS) of IPMP isolated from volatiles collected from headspace of five live *H. axyridis* (Part A) and pure standard (Part B). MS scan mode: total ion scan mode.

Table 3
Repeatability and reproducibility for HS-SPME–GC–MS

Conc. (μg/L)	IPMP	SBMP	IBMP
Repeatability (%)[a]			
0.05	1.6	3.8	3.6
0.01	5.0	2.9	3.9
0.005	3.2	3.2	6.5
0.0005	5.2	5.1	8.7
0.0001	1.6	0.1	3.9
Reproducibility (%)[b]			
0.0001	7.8	7.1	7.7

[a] $n = 3$.
[b] $n = 10$. Measurement taken on different days.

low. Seifert et al. [8] found that the isopropyl and isobutyl methoxypyrazines had an odor detection threshold in the order of 2 ng L^{-1} in water. Seifert et al. [8] reported that shortening the chain from butyl/propyl to ethyl group weakened the odor strength. Further shortening to methyl group resulted in further weakening of odor threshold to approximately 4000 ng L^{-1} in water [8]. It is therefore reasonable to assume that the MPs discussed in this manuscript have a similar odor detection threshold.

3.6. Validation of analytical method

Repeatability and reproducibility of the HS-SPME–GC–MS method were expressed as RSDs of the three MPs. Repeatability was evaluated by analysis of three MPs at five different concentration levels of 0.1, 0.5, and 5 ng L^{-1}, 0.01 and 0.05 μg L^{-1} with three replicates for each concentration. The reproducibility experiment was performed at lowest concentration (0.1 ng L^{-1}) with ten replicates in three different days. The RSDs at different concentrations were less than 8.7% (Table 3). For the 0.1 ng L^{-1} concentration, the intra- and inter-day precision for the three MPs were <3.9 and 7.8%.

The linearity ranges, linear regression equation and R^2 for HS-SPME–GC–MS method are summarized in Table 4. The calibration curve for three MPs was linear over the concentration range of 0.1 ng L^{-1}–0.05 μg L^{-1}, with R^2 value equal to 0.9984 for IPMP, 0.9976 for SBMP and 0.9951 for IBMP, respectively. Headspace sample recovery was tested using 10 ng L^{-1} standard mixture for three MPs with two consecutive 24 h extractions with HS-SPME. This was done to test the assumption that the MPs are volatized to headspace and that headspace extractions are exhaustive under the optimized SPME extraction conditions.

Table 4
Equations of calibration curve, R^2 and MDLs for target methoxypyrazines

Compound	Conc. (μg L^{-1})	Equation	R^2	MDL with narrow heart-cut (ng L^{-1})	MDL with full heart-cut (ng L^{-1})
HS-SPME–GC–MS (five standard concentration)					
IPMP	0.0001–0.05	$y = 3.63E+06x - 1.67E03$	0.9984	0.022	0.048
SBMP	0.0001–0.05	$y = 2.94E+06x - 4.62E02$	0.9976	0.020	0.044
IBMP[a]	0.0001–0.05	$y = 4.81E+06x$	0.9951	0.022	0.036
Direct injection-GC–MS (six standard concentration)					
IPMP	10–10,000	$y = 1.74E02x - 2.95E04$	0.9972	n/a	n/a
SBMP	10–10,000	$y = 1.44E02x - 2.26E04$	0.9974	n/a	n/a
IBMP[a]	10–10,000	$y = 1.96E02x$	0.9945	n/a	n/a

n/a: not available; MDL: method detection limit.
[a] All measured concentrations of MPs from live *H. axiridis* were above the estimated MDL and below the lowest linear concentration, so calibration curve for IBMP was forced through origin.

The sample mass recovery was 96% for IPMP, 99% for SBMP and 98% for IBMP, respectively, in the first extraction. The RSDs varied from 2.4 to 6.2% ($n=3$) for three MPs for the first extraction and from 2.6 to 9.2% ($n=3$) for the second extraction.

The sensitivity of HS-SPME method was compared with direct injection method. The direct injection method was also used to calibrate MS detector. The linear regression equations and R^2 for direct injection calibration curves were based on six standard concentrations (10, 50, 100, 500, 1000, and 10,000 $\mu g L^{-1}$) and are also presented in Table 4. The R^2 values were 0.9972 for IPMP, 0.9974 for SBMP, and 0.9945 for IBMP, respectively. It is interesting to mention that the difference between the lowest concentrations of standards used for the HS-SPME and direct injection method with comparable MS response was approximately 5 orders of magnitude, i.e., 0.1 $ng L^{-1}$ versus 10 $\mu g L^{-1}$.

The MDLs for HS-SPME–GC–MS were estimated using Eq. (1) and are listed in Table 4. The MDLs were estimated and compared for the two approaches used to isolate MPs; (a) HS-SPME–MDGC–MS, i.e., with narrow heart-cut and (b) HS-SPME–GC–MS with full heart-cut. The MDLs were estimated based on the experiment with 10 replicate HS-SPME extractions of the MPs at the lowest concentration (0.1 $ng L^{-1}$) of linearity range using Eq. (1). The detection limit obtained for HS-SPME–MDGC–MS with narrow heart-cut method was always lower for each MP and improved by 52.2, 52.4, and 38.9% for IPMP, SBMP, and IBMP, respectively. This improvement is likely due to the reduction of interferences with the introduction of narrow heart-cuts in multidimensional mode.

3.7. Estimation of IPMP, SBMP, and IBMP releases per beetle mass and per beetle

The MDGC–MS–O approach was used to quantify MPs released to headspace using SPME and *in vivo* sampling. The estimated amounts of three MPs emitted from live *H. axyridis* are presented in Table 5. The average amounts of three MPs per beetle mass (for $n=8$ replicates for orange beetles, with each replicate comprised of five beetles in a 40 mL vial) were 8.0569 $ng g^{-1}$ for IPMP, 3.1738 $ng g^{-1}$ for SBMP and 0.0126 $ng g^{-1}$ for IBMP, respectively. The average amounts of three MPs per beetle mass (for $n=2$ replicates for yellow beetles, with each replicates comprised of five yellow beetles in a 40 mL vial) were 0.4111 $ng g^{-1}$ for IPMP, 0.6191 $ng g^{-1}$ for SBMP and 0.0055 $ng g^{-1}$ for IBMP, respectively. For pooled orange and yellow beetles, the average were 4.2340 $ng g^{-1}$ for IPMP, 1.8965 $ng g^{-1}$ for SBMP and 0.0091 $ng g^{-1}$ for IBMP, respectively. The amounts of MPs estimated as mass per beetle (Table 5) are consistent with the odor intensity recorded by three panelists. IPMP had the strongest odor and was the most abundant MP (Table 5). The dominance of IPMP among other MPs emitted from *H. axyridis* is consistent with observations by Cudjoe et al. [3] who reported that IPMP had the greatest aroma intensity due to its higher gas emissions from frozen/thawed beetles.

Although it was not our goal to determine the relationship between adult color and MP concentrations, we did observe

Table 5
Estimated amounts ($ng g^{-1}$ and ng/beetle) of three methoxypyrazines emitted to vial headspace from live *H. axiridis* and detected by HS-SPME–MDGC–MS

Compound	Orange beetles ($n=8$ vials with 5 beetles for each)				Yellow beetles ($n=2$ vials with 5 beetles for each)				All beetles ($n=10$ vials with 5 beetles for each)
	Mean ($ng g^{-1}$)	RSD (%)	Min ($ng g^{-1}$)	Max ($ng g^{-1}$)	Mean ($ng g^{-1}$)	RSD (%)	Min ($ng g^{-1}$)	Max ($ng g^{-1}$)	Mean ($ng g^{-1}$)
IPMP	8.0569	116.83	1.3383	29.4338	0.4111	19.74	0.3538	0.4685	4.2340
SBMP	3.1738	82.45	0.4370	6.5493	0.6191	62.38	0.3460	0.8921	1.8965
IBMP[a]	0.0126	42.89	0.0058	0.0230	0.0055	50.71	0.0035	0.0074	0.0091
Compound	**Orange beetles ($n=8$ vials with 5 beetles for each)**				**Yellow beetles ($n=2$ vials with 5 beetles for each)**				**All beetles ($n=10$ vials with 5 beetles for each)**
	Mean (ng/beetle)	RSD (%)	Min (ng/beetle)	Max (ng/beetle)	Mean (ng/beetle)	RSD (%)	Min (ng/beetle)	Max (ng/beetle)	Mean (ng/beetle)
IPMP	0.3080	128.88	0.0524	1.2244	0.0143	23.70	0.0119	0.0167	0.1612
SBMP	0.1124	79.53	0.0161	0.2398	0.0251	69.89	0.0127	0.0374	0.0688
IBMP[a]	0.0006	51.64	0.0003	0.0012	0.0002	47.14	0.0002	0.0003	0.0004

[a] All measured concentrations of MPs from live *H. axiridis* were above the estimated MDL and below the lowest linear concentration, so calibration curve for IBMP was forced through origin.

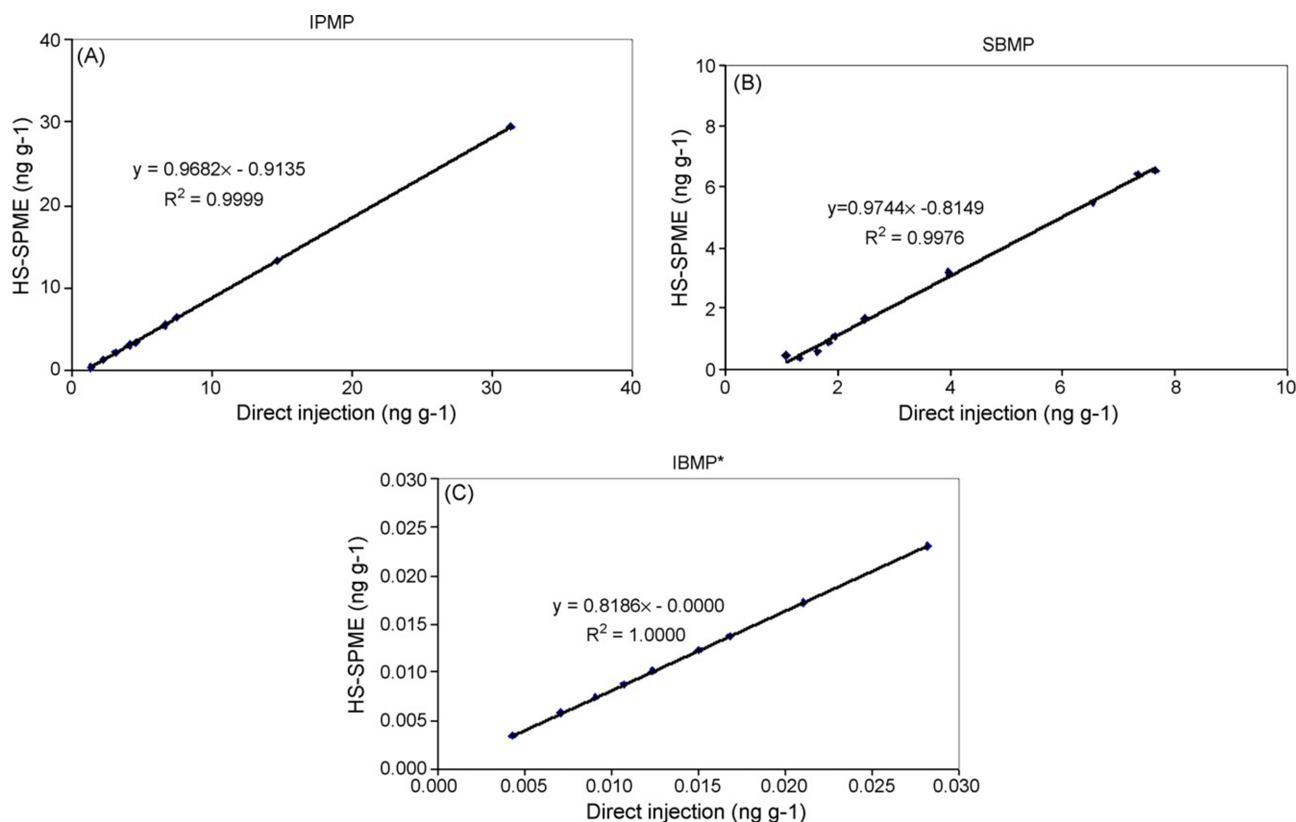

Fig. 9. Comparison of MPs emissions from live *H. axiridis* to headspace estimated with two different methods of calibration: HS-SPME–GC–MS with heart-cut and by direct injection in GC–MS with heart-cut. (Part A) IPMP; (Part B) SBMP; (Part C) IBMP. MS scan mode: SIM mode. *All measured concentrations of MPs from live *H. axiridis* were above the estimated MDL and below the lowest linear concentration; calibration curve for IBMP was forced through origin.

evidence of such a relationship (Table 5). There is a great variation in the color and marking patterns of *H. axyridis* [32]. In North America, adults vary from a bright red to a pale orange color with a range in the number and location of black spots. Outside of North America, phenotypic variation is even greater including black morphs with orange to red spots. This aposematic coloration is determined both by genetic factors and the influence of the larval diet [33]. Although the relationship is not clear between color intensity of adult *H. axyridis* and MP content [34], we separated beetles by apparent color to reduce a possible source of variation. Interestingly, the average concentration of all MPs (IPMP, SBMP, and IBMP) were lowest in the yellow replicates and the highest in the orange replicates (mean ± standard deviation; $1.036 \pm 0.46\,\text{ng g}^{-1}$ and $11.243 \pm 10.32\,\text{ng g}^{-1}$ for yellow and orange beetles, respectively). Although this represents a small, unbalanced data set, we did observe a significant difference between these two means ($t = 2.89$, df = 9, $P = 0.02$; PROC TTEST, SAS 2002) [35]. If there is a relationship between adult color and MP concentrations, this would help explain differences in our estimates of *H. axyridis* MP concentrations versus those of other investigators that did not account for this potential source of variation [3].

The estimated emissions of three MPs were also compared for the two methodologies (direct injection versus HS-SPME; Fig. 9). Ten vials with five live beetles for each vial were analyzed by both methods. The individual data, correlation and regression analyses are presented in Fig. 9 (Parts A, B and C). All correlation coefficients were greater than 0.99, indicating good linearity, i.e., for IPMP ($R^2 = 0.9999$) and SBMP ($R^2 = 0.9976$). For IBMP, linear relationship was less robust ($R^2 = 0.7174$, data not shown in Fig. 9). This is likely due to the uncertainties associated with measured low concentrations near the estimated MDL. However, if the calibration curve for IBMP is plotted through the origin, there was a linear relationship for IBMP ($R^2 = 1.0000$) between two methods (direct injection versus HS-SPME). The slopes of regression line for three MPs were greater than 0.82 indicating that both methods were in good agreement.

4. Conclusions

In vivo HS-SPME combined with multidimensional GC–MS–O has a great potential for investigating links between specific chemicals released by insects and their characteristic odors. In this research, 50/30 μm DVB/Carboxen/PDMS SPME fiber was used to extract headspace volatiles released by live *H. axyridis*. Thirty-eight compounds were identified in headspace of live *H. axyridis* including four characteristic odorous compounds—DMMP, IPMP, SBMP and IBMP. We detected a previously unidentified MP (DMMP) that appears to be also a component of *H. axyridis*'s odor. We also provided conclusive evidence that IPMP released within the headspace of living *H. axyridis* is responsible for their characteristic odor. Quantification of three MPs (IPMP, SBMP and IBMP) emitted from live beetles was performed using external calibration

curves by HS-SPME–MDGC–MS. Linear relationships (R^2 was >0.9951 for all 3 MPs) were observed over a concentration range from 0.1 ng L^{-1} to 0.05 μg L^{-1}. The MDLs were estimated at 0.022, 0.020, and 0.022 ng L^{-1} for IPMP, SBMP, and IBMP, respectively. These MDLs obtained with multidimensional GC–MS with narrow heart-cut approach represent 52.2, 52.4, and 38.9% improvement compared to GC–MS with full heart-cut approach. For the 0.1 ng L^{-1} concentration, the intra- and inter-day precision for the three MPs were less than 3.9 and 7.8%. Using the HS-SPME–MDGC–MS method we estimated that live *H. axyridis* per beetle body mass released 4.2340 ng g^{-1} of IPMP, 1.8965 ng g^{-1} of SBMP and 0.0091 ng g^{-1} of IBMP. We observed a significant correlation between MP emissions and beetle color, with orange beetles releasing more MP than yellow beetles. Although this observation requires further experiments with a more balanced sample, it represents a previously undocumented source of variability in MP from *H. axyridis*. We conclude that HS-SPME–MDGC–MS–O is a novel, fast and reliable methodology for the determination of characteristic odorants (MPs) emitted from live *H. axyridis*. This method has potential to be used for rapid *in vivo* determination of odor-causing chemicals associated with emissions of volatiles from other insects.

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