Why do Ladybugs Smell Bad? In-vivo Quantification of Odorous Insect Kairomones with SPME and Multidimensional GC-MS-Olfactometry

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Keywords
chemical sensors, electrochemical sensors, mass spectroscopy

Disciplines
Agriculture | Bioresource and Agricultural Engineering

Comments
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Why do Ladybugs Smell Bad?

In-vivo Quantification of Odorous Insect Kairomones with SPME and Multidimensional GC-MS-Olfactometry

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Abstract. Winemakers, small fruit growers, and homeowners are concerned with noxious compounds released by multicolored Asian ladybird beetles (Harmonia axyridis, Coleoptera: Coccinellidae). 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. 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 (R2 >0.9958 for all 3 MPs) between MS response and concentration of 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 approach for three MPs were estimated to be between 0.020 ng L-1 to 0.022 ng L-1. This methodology is applicable for in vivo determination of odor-causing chemicals associated with emissions of volatiles from insects.

Keywords: Harmonia axyridis, SPME, Multidimensional GC–Olfactometry, Odor, Methoxypyrazines

PACS: 01.30.Cc

INTRODUCTION

The recent invasion and establishment of Harmonia axyridis (Coleoptera: Coccinellidae) in North America has resulted in a pest on several fronts. 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 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 MP.2 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 level of 2 ng L-1 in water.3

The larvae and adults are primarily predators and have been considered a significant source of biological control for another invasive pest, the soybean aphid, Aphis glycines (Hemiptera: Aphididae). 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. axyridis. When processed into wine, MPs released from lady beetles have been identified as a fouling agent.4 Allen et al. (1998) reported lower odor detection thresholds in white wine compared with red wine.4 Pickering et al. found H. axyridis released MPs, particularly IPMP was the agent responsible for the wine taint.5

The concentration of MPs released by lady beetles (Coccinellids) is in the order of pg/beetle [6] and ng L-1 in wine.4 Therefore it is necessary to develop highly sensitive extraction and analysis methods for qualitative and quantitative purpose at such low levels.

In this research, headspace (HS) SPME was used for extraction of volatiles released by live H. axyridis. This approach combines rapid sampling and sample preparation, olfactometry and multidimensional GC separation with conventional MS detector. 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 to (2) determine the amounts of those characteristic odorants emitted from live H. axyridis.
EXPERIMENTAL AND METHODS

Standards and Solutions

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 methanol. The external calibration standard solutions ranged from 0.1 ng L\(^{-1}\) to 0.05 µg L\(^{-1}\).

Isolation of Characteristic Odorants with Multidimensional GC-MS-O

Multidimensional GC-MS-olfactometry (MDGC-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. 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 30 m × 0.53 mm 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™ V. 6.00 and AromaTrax™ V. 6.63 (Microanalytics, Round Rock, TX, USA) and ChemStation™ (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. Mass to charge ratio (m/z) range was set between 33 and 280. Spectra were collected at 6 scans sec\(^{-1}\) and electron multiplier voltage was set to 1400 V. The detection of trace three MPs was carried out using selected ion monitoring. m/z =137, 138 and 124 were used for quantification for IPMP, SBMP and IBMP, respectively. The MS detector was auto-tuned every day.

Sensory evaluations were made through the sniff port equipped with two capillary columns. 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). 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.

In Vivo Headspace SPME of Compounds Released by Live \(H. \)Axyridis

\(H. \)axyridis were collected in Ames, Iowa in September, 2005, February and August 2006. Multiple sets of randomly-selected five live \(H. \)axyridis were then placed in screw-capped vials (40 mL, Supelco, Bellefonte, PA, USA) sealed with a PTFE-lined silicone septum and used for in vivo HS-SPME. Each vial with beetles was allowed to equilibrate for 24 h before HS-SPME at 30 °C. Headspace samples from life beetles only were considered for analyses, i.e., if the beetles died during sampling, the samples were discarded.

RESULTS AND DISCUSSION

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. In order to identify the characteristic odors from live \(H. \)axyridis, three panelists analyzed headspace volatiles released by live \(H. \)axyridis through sniff port. The panelists were consistent identifying four ‘characteristic’ odors, and also describing them as ‘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 other MPs. 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.

SBMP was positively identified by matching the retention time of 40 standard compounds and matching mass spectrum of unknown compound with BenchTop/PBM.
‘peanut’, and ‘potato’. One compound was consistently tagged by all panelists with the characteristic odor, i.e., ‘roasted peanuts’ and later tentatively identified as DMMP by the mass spectrum match greater than 90 % with BenchTop/PBM library. Seifert et al. reported ‘roasted peanut’ aroma and tentatively associated it with methyl MPs without specifically pointing to DMMP.\(^3\) The release of IPMP, SBMP, and IBMP from dead beetles has been reported in previous studies.\(^2\) However, we are not aware of any previous report of DMMP released by \textit{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 \textit{H. axyridis}.

Previous studies suggested that IPMP is the most important component of \textit{H. axyridis}’s aroma. Cudjoe et al. found IPMP was the most abundant MPs released by dead \textit{Coccinella septempunctata}, \textit{Harmonia axyridis} and \textit{Hippodemia convergens} lady beetles (Coccinellidae).\(^2\) Pickering et al. 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 \textit{H. axyridis}.\(^5\) Pickering et al. also found that IPMP is responsible for the distinctive sensory characteristics of \textit{H. axyridis} contaminated wines and found significant positive correlations between IPMP concentration and specific aroma attributes in wines.\(^5\)

**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. 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. The description of odor released by \textit{H. axyridis} that is often described as ‘green bell pepper’, ‘roasted peanuts’ or ‘green peas’. 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 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 \textit{H. axyridis} is shown in Figure 1. As can be seen in Figure 1, the separation of IPMP and 2-ethyl-1-hexanol was much improved even though it was not a baseline separation.

**FIGURE 1.** Separations for MPs from the headspace of 5 live \textit{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.

**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 1. The average amounts of three MPs per beetle mass (for \( n = 8 \) replicates for red beetles, with each replicate comprised of five beetles in a 40 mL vial) were 8.0569 ng g\(^{-1}\) for IPMP, 3.1680 ng g\(^{-1}\) for SBMP and 0.0811 ng g\(^{-1}\) for IBMP, respectively. The average amounts of three MPs per beetle mass (for \( n = 2 \) replicates for orange beetles, with each replicate comprised of five orange 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 red and orange 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.

**TABLE 1.** Estimated amounts (ng g\(^{-1}\) and ng per beetle) of three methoxypyrazines emitted to vial headspace from live *H. axyridis* and detected by HS-SPME-MDGC-MS.

<table>
<thead>
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<th>Red beetles (n = 8)</th>
<th>Orange beetles (n = 2)</th>
<th>All beetles (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>(ng g(^{-1}))</td>
<td>(ng g(^{-1}))</td>
<td>(ng g(^{-1}))</td>
</tr>
<tr>
<td>IPMP</td>
<td>8.06</td>
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<td>4.2340</td>
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<tr>
<td>SBMP</td>
<td>3.17</td>
<td>0.619</td>
<td>1.8965</td>
</tr>
<tr>
<td>IBMP</td>
<td>0.01</td>
<td>0.0065</td>
<td>0.0091</td>
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<tr>
<td><strong>Mean</strong></td>
<td>(ng)</td>
<td>(ng)</td>
<td>(ng)</td>
</tr>
<tr>
<td>IPMP</td>
<td>0.308</td>
<td>0.014</td>
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</tr>
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<td>SBMP</td>
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<td>IBMP</td>
<td>0.0006</td>
<td>0.0002</td>
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</table>

**CONCLUSIONS**

*In vivo* HS-SPME combined with multidimensional GC-MS-O has a great potential for investigations of links between specific chemicals released by insects and the 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 the first evidence that IPMP is released to air and is also responsible for the characteristic odor of live *H. axyridis*. Quantification of three MPs, i.e., IPMP, SBMP and IBMP, emitted from live beetles was performed using external calibration curves by HS-SPME-MDGC-MS. Linear relationships (R\(^2\) was > 0.9958 for all 3 MPs) was observed over a concentration range from 0.1 ng L\(^{-1}\) to 0.05 µg L\(^{-1}\). The MDLs were estimated at 0.022 ng L\(^{-1}\), 0.020 ng L\(^{-1}\), 0.022 ng L\(^{-1}\) for IPMP, SBMP, and IBMP, respectively. These MDLs obtained with multidimensional GC-MS approach represent 52.2%, 52.4%, and 38.9% improvement compared to GC-MS 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 %. The HS-SPME-MDGC-MS method was applied to determine the amounts of three MPs emitted to headspace from live *H. axyridis* per beetle body mass and the average amounts of MPs were 4.2340 ng g\(^{-1}\) for IPMP, 1.8965 ng g\(^{-1}\) for SBMP and 0.0091 ng g\(^{-1}\) for IBMP, respectively.

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