Iowa State University
Digital Repository

Agricultural and Biosystems Engineering Publications

Agricultural and Biosystems Engineering

4-13-2007

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

Lingshuang Cai
Iowa State University

Jacek A. Koziel
Iowa State University, koziel@iastate.edu

Matthew E. O'Neal
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/abe_eng_pubs

Part of the Agriculture Commons, Bioresource and Agricultural Engineering Commons, and the Entomology Commons

The complete bibliographic information for this item can be found at https://lib.dr.iastate.edu/abe_eng_pubs/927. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.

This Article is brought to you for free and open access by the Agricultural and Biosystems Engineering at Iowa State University Digital Repository. It has been accepted for inclusion in Agricultural and Biosystems Engineering Publications by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

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

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 (R2 > 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.

Keywords

Harmonia axyridis, Headspace solid-phase microextraction, Multidimensional gas chromatography–olfactometry, Odor, Methoxypyrazines

Disciplines

Agriculture | Bioresource and Agricultural Engineering | Entomology

Comments

This is a manuscript of an article published as Cai, Lingshuang, Jacek A. Koziel, and Matthew E. O'Neal. "Determination of characteristic odorants from Harmonia axyridis beetles using in vivo solid-phase microextraction and multidimensional gas chromatography–mass spectrometry–olfactometry." *Journal of Chromatography A* 1147, no. 1 (2007): 66-78. DOI: 10.1016/j.chroma.2007.02.044. Posted with permission.

Creative Commons License

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 4.0 License.

This article is available at Iowa State University Digital Repository: https://lib.dr.iastate.edu/abe_eng_pubs/927

1

2

3

4

5 **Determination of characteristic odorants from *Harmonia axyridis***

6 **beetles using *in vivo* solid-phase microextraction and multidimensional**

7 **gas chromatography mass spectrometry – olfactometry**

8

9 Lingshuang Cai,[1] Jacek A. Koziel,*[1] Matthew E. O'Neal[2]

10 [1]Department of Agricultural & Biosystems Engineering,

11 Iowa State University, Ames, IA, USA

12 *corresponding author, phone 515-294-4206; fax 515-294-4250; e-mail:

13 koziel@iastate.edu.

14 [2]Department of Entomology, Iowa State University, Ames, IA, USA

15

16 ## Abstract

17 Homeowners, small fruit growers, and wine makers are concerned with noxious
18 compounds released by multicolored Asian ladybird beetles (*Harmonia axyridis*,
19 Coleoptera: Coccinellidae). A new method based on headspace solid phase
20 microextraction (HS-SPME) coupled with multidimensional gas chromatography mass
21 spectrometry – olfactometry (MDGC-MS-O) system was developed for extraction,
22 isolation and simultaneous identification of compounds responsible for the characteristic
23 odor of live *H. axyridis*. Four methoxypyrazines (MPs) were identified in headspace
24 volatiles of live *H. axyridis* as those responsible for the characteristic odor: 2,5-dimethyl-
25 3-methoxypyrazine (DMMP), 2-isopropyl-3-methoxypyrazine (IPMP), 2-sec-butyl-3-
26 methoxypyrazine (SBMP), and 2-isobutyl-3-methoxypyrazine (IBMP). To the best of our
27 knowledge this is the first report of *H. axyridis* releasing DMMP and the first report of
28 this compound being a component of the *H. axyridis* characteristic odor. Besides the
29 MPs, thirty four additional compounds were also identified. Quantification of three MPs
30 (IPMP, SBMP and IBMP) emitted from live *H. axyridis* were performed using external
31 calibration with HS-SPME and direct injections. A linear relationship (R^2 >0.9958 for all
32 3 MPs) between MS response and concentration of a standard was observed over a
33 concentration range from 0.1 ng L^{-1} to 0.05 µg L^{-1} for HS-SPME-GC-MS. The method
34 detection limits (MDL) based on multidimensional GC-MS with heart-cut approach for
35 three MPs were estimated to be between 0.020 ng L^{-1} to 0.022 ng L^{-1}. This represents a
36 38.9% to 52.4% improvement in sensitivity compared to GC-MS without heart-cut
37 method. This methodology is applicable for *in vivo* determination of odor-causing
38 chemicals associated with emissions of volatiles from insects.

39 *Keywords*: *Harmonia axyridis*; Headspace solid phase microextraction;
40 Multidimensional gas chromatography-olfactometry; Odor; Methoxypyrazines

41 ## 1. Introduction

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

54 The larvae and adults of *H. axyridis* are primarily predators and have been
55 considered a significant source of biological control for another invasive pest, the
56 soybean aphid, *Aphis glycines* (Hemiptera: Aphididae). However the feeding habits of *H.*
57 *axyridis* in North America are more cosmopolitan, with reports of fall feeding on several
58 fruits [2]. The impact of this feeding by adult *H. axyridis* as a significant source of yield
59 loss is not clear. A greater threat may be a loss in fruit quality, especially grapes, when
60 harvested fruit is contaminated with adult *H. axyiridis*. When processed into wine, MPs
61 released from lady beetles have been identified as a fouling agent [9-12]. Allen et al.

62 (1998) reported lower odor detection thresholds in white wine compared with red wine
63 [9]. Pickering et al. [10] found *H. axyridis* released MPs, particularly IPMP was the
64 agent responsible for the wine taint.

65 The concentration of MPs released by lady beetles (coccinellids) is in the order of
66 pg/beetle [5] and ng L^{-1} [10-13] in wine. Therefore it is necessary to develop highly
67 sensitive extraction and analysis methods for qualitative and quantitative purpose at such
68 low levels. Comparison of sampling and analytical methods in previous studies to
69 characterize odorants released by lady beetles is shown in Table 1 [2,3,10,12,14,15]. Gas
70 chromatography has been commonly employed for this purpose due to the volatility of
71 MPs. However, the detection of volatiles at low concentrations from complex matrixes
72 first requires an extraction and a preconcentration step in sample preparation procedures.
73 In addition, processing large numbers of insects is often required to determine low levels
74 of certain volatiles. Extraction by cation-exchange resin, liquid-liquid extraction [16]; C-
75 18 SPE extraction [10,12] and solvent extraction [2] were used to concentrate MPs from
76 various sample matrices in previous studies. Recently, a simple one step sampling and
77 sample preparation technique, SPME, was used for determination of MPs from wine or
78 grape juice headspace [17-20]. SPME is solventless, reusable sampling/sample
79 preparation technique suited for rapid qualitative and quantitative analyses [21]. It has
80 been used to non-invasively sample aromas and fragrances emitted from plants and
81 insects [22].

82 Our goal was to validate the use of headspace (HS) SPME for extraction of
83 volatiles released by live *H. axyridis*. The same HS-SPME-MDGC-MS-Olfactometry
84 approach has been successfully used to characterize livestock odors [23-26]. This

85 approach combines rapid sampling and sample preparation, olfactometry and
86 multidimensional GC separation with conventional MS detector. Simultaneous isolation,
87 identification, and analysis of volatiles and their corresponding odors improve separation
88 and sensitivity compared to conventional, one column GC. The objective of this study
89 was to (1) confirm if MPs are the sole source of noxious odors from *H. axyridis* using a
90 novel approach - multidimensional GC coupled with olfactometry and to (2) determine
91 the amounts of those characteristic odorants emitted from live *H. axyridis*.
92

93 ## 2. Materials and Methods

94

95 *2.1. Standards and solutions*

96 Reference standards used for identification were purchased from Sigma-Aldrich
97 (St. Louis, MO, USA) and included 1,4-pentadiene (99%, purity), acetone (99%), heptane
98 (99%), 2-butanone (99%), diacetyl (97%), 2-pentanone(99%), octane (98%), 2-methyl-3-
99 buten-2-ol (98%), methyl benzene (99.9%), nonane (99%), isoamyl alcohol (99%), alpha-
100 pinene (98%), camphene (95%), 3-hydroxy-2-butanone (99%), limonene (99%), octanal
101 (99%), 6-methyl-5-hepten-2-one (99%), acetic acid (99.7%), 1,3-dichlorobenzene (98%),
102 nonanal (95%), 2-isopropyl-3-methoxypyrazine (97%, IPMP), 2-ethyl-1-hexanol (99.6%),
103 propanoic acid (99%), benzaldehyde (99%), dihydro-3-methyl- 2[3H]-furanone (98%), 2-
104 sec-butyl-3-methoxypyrazine (99%, SBMP), 2-isobutyl-3-methoxypyrazine (97%,
105 IBMP), isovaleric acid (99%), 1-borneol (95%), benzenemethanol (99%), phenol (99%),
106 and indole (99%), respectively.

107 The three standards (IPMP, SBMP and IBMP) were used for quantification of the
108 amount of MPs emitted from live beetles. An individual standard solution of 1 mg mL^{-1}
109 of each MP was prepared in certified A.C.S.-grade methanol (Fisher Scientific,
110 Pittsburgh, PA, USA). A combined standard solution containing all the analytes was
111 prepared with each individual solution and subsequently diluted with methanol within a
112 volumetric flask, sealed with parafilm and covered with aluminum foil to avoid photo-
113 degradation [27]. All the solutions were stored in dark at 4 ° C until use. Ultrapure-grade
114 water from a high purity water system (Culligan Water Conditioning, Lexington, KY,
115 USA) with conductivity 18 MΩ was used in all cases. Standard solutions used in further
116 studies were prepared fresh by diluting different amounts of the standard solution with
117 pure water to the required concentrations. The external calibration standard solutions
118 ranged from 0.1 ng L^{-1} to 0.05 μg L^{-1} and were made by dilution of the stock solutions in
119 water using optimized HS-SPME conditions. A certain volume of the standard solution
120 were placed in 40 mL vials with a stir bar (Fisher Scientific, Pittsburgh, PA, USA) and
121 prefilled with 20 mL of pure water and 3 g NaCl from Sigma-Aldrich (St. Louis, MO,
122 USA). The HS-SPME extractions were preformed at 25 ºC with 24 h extraction time and
123 constant stirring. Samples were run in triplicates.
124

125 *2.2. Isolation of characteristic odorants with MDGC-MS-O system*

126 Multidimensional GC-MS-olfactometry (MDGC-O) system (Microanalytics, Round
127 Rock, TX, USA) built on a 6890N GC / 5973 MS platform (Agilent Inc., Wilmington,
128 DE, USA) were used for all analyses. This system allows for the simultaneous
129 identification and analysis of chemicals and corresponding odors. The system was

130 equipped with two columns in series connected by a Dean's switch. The non-polar pre-
131 column was 12 m, 0.53 mm i.d.; film thickness, 1 µm with 5% phenyl
132 methylpolysiloxane stationary phase (SGE BP5) and operated with constant pressure
133 mode at 8.5 psi. The polar analytical column was a 30 m × 0.53 mm fused silica capillary
134 column coated with poly (ethylene glycol) (WAX; SGE BP20) at a film thickness of 1
135 µm. The column pressure was constant at 5.8 psi. Both columns were connected in series.
136 System automation and data acquisition software were MultiTrax™ V. 6.00 and
137 AromaTrax™ V. 6.63 (Microanalytics, Round Rock, TX, USA) and ChemStation™
138 (Agilent, Santa Clara, CA, USA). The general run parameters used were as follows:
139 injector, 260 °C; FID, 280 °C, column, 40 °C initial, 3 min hold, 7 °C min^{-1}, 220 °C final,
140 10 min hold; carrier gas, GC-grade helium. The GC was operated in a constant pressure
141 mode where the mid-point pressure, i.e., pressure between pre-column and column, was
142 always at 5.8 psi and the heart-cut sweep pressure was 5.0 psi. The FID connected to the
143 pre-column was maintained at 280 °C with a H_2 flow rate of 35 mL min^{-1}, an air flow rate
144 of 350 mL min^{-1}, and the makeup N_2 flow rate of 10 mL min^{-1}. The FID data acquisition
145 rate was 20 Hz. Mass to charge ratio (m/z) range was set between 33 and 280. Spectra
146 were collected at 6 scans sec^{-1} and electron multiplier voltage was set to 1400 V. The
147 detection of trace three MPs was carried out using selected ion monitoring. Mass
148 channels were m/z = 124, 137 and 152 for IPMP, m/z =124, 138 and 151 for SBMP, m/z
149 = 94,124 and 151 for IBMP with 50 ms dwell times. Therefore, m/z =137, 138 and 124
150 were used for quantification for IPMP, SBMP and IBMP, respectively. The MS detector
151 was auto-tuned every day. SIM mode was used for all quantification analysis including

152 validation of analytical method; estimation of IPMP, SBMP, and IBMP releases per
153 beetle mass and per beetle and direct injection method.
154 Heart-cut valve based on Dean's switch concept was located between the pre-
155 column and analytical column. In such a dual column system, the heart-cut valve was
156 used to transfer specific pre-separated retention regions with characteristic *H. axyridis*
157 odor from the pre-column (and the entire sample matrix) to the analytical column.
158 Transferring only the selected odor-causing compounds to the analytical column was
159 done to improve the quality of olfactometry and chemical analyses by reducing the
160 background signals from other odorless or less relevant compounds. In this research,
161 finding the specific odor-causing compounds was first accomplished by GC-O analyses
162 on the pre-column with trained panelists at the sniff port. The specific retention time
163 regions with characteristic odors were selected based on reproducible odor detection
164 start/end times. Then, in the subsequent sample analyses, the heart-cut times were set
165 with the MultiTrax™. In the follow up GC run, only the narrow segments of
166 chromatographic effluent that contained the characteristic odors of interest from the pre-
167 column separation were then transferred to the analytical column and analyzed
168 simultaneously with the MS detector and a trained panelist at the sniff port.
169 Sensory evaluations were made through the sniff port equipped with two capillary
170 columns. Only one of them was allowed to deliver a sample to a panelist depending on
171 the instrument mode. The split ratio between the MS and the sniff port was 1:3. The
172 temperature for the sniff port capillaries was set to 220 °C to eliminate condensation. In
173 addition, humidified air (Certified breathing air grade, 99.995% purity, Praxair, Inc.,
174 Danbury, CT, USA) was constantly delivered to the sniff port at 8.0 psi. This was done to

175 maintain a constant humidity level for the panelists' mucous nasal membranes. The tip of
176 the sniff port was equipped with a glass nose cone (SGE, Austin, TX, USA). The
177 olfactory responses of panelists were recorded as aromagrams using Aromatrax software
178 by applying an odor tag (odor event) to a peak or a region of the chromatographic
179 separation. The odor tag (odor event) consisted of editable odor character descriptors, an
180 odor event time span and perceived odor intensity. The aroma intensity was measured
181 with a 0-100 % point scale (100% indicated strongest odor and 0% indicated no odor).
182 Panelists with extensive GC-O experience were trained on standard methoxypyrazines
183 and practiced extensively with live beetle samples before controlled experiments. Three
184 trained panelists analyzed headspace volatiles from live *H. axyridis*. Panelist responses
185 were compared based on odor character and odor intensity associated with separated
186 compounds.
187
188 *2.3. Data analysis*
189 Three sets of signals were generated for each sample including the total ion
190 chromatogram (TIC), the FID signal, and the Aromagram. The TIC was generated from
191 gases extracted from headspace samples by the MSD in HC mode (GC-MS-O) including
192 full HC (the heart-cut valve was open between 0.05 and 35 min run-time) and HC (the
193 heart-cut valve was open on specific time range of interest based on GC-FID-O mode).
194 Each TIC consisted of mass spectra for each compound and were combined and plotted
195 as a function of retention time. Chromatogram for the FID contained a non-zero signal
196 when the instrument was operated in no HC mode (GC-FID-O). For the no HC mode,
197 the gas sample passed only through the non-polar precolumn and entered the FID without

198 going into the polar column. Compounds were separated in the precolumn and the
199 chromatogram of the sample was plotted in the FID graph. Based on this 'screening' in
200 GC-FID-O mode, the heart-cut range was determined by recognizing what are needed
201 further separation in multidimensional GC-MS-O mode.
202 Compounds were identified with 3 sets of criteria: (1) matching of the retention
203 time on the MDGC capillary column with the retention time of pure compounds run as
204 standards, (2) matching mass spectrums (>70%) of unknown compounds with
205 BenchTop/PBM (Palisade Mass Spectrometry, Ithaca, NY, USA) MS library search
206 system and spectrums of pure compounds, and (3) matching odor character.
207
208 *2.4.* In vivo *headspace SPME of compounds released by live* H. axyridis.
209 *H. axyridis* were collected as needed in Ames, Iowa in September 2005, February
210 and August 2006. *H. axyridis* were maintained in a 500 mL ventilated plastic bottle
211 containing cotton swabs with water at room temperature (~20 °C) prior to experiments.
212 Field collected beetles were separated by color, into two groups of either yellow or
213 orange. Multiple sets of randomly-selected five live from each group of *H. axyridis* were
214 then placed in screw-capped vials (40 mL, Supelco, Bellefonte, PA, USA) sealed with a
215 polytetrafluoroethylene (PTFE)-lined silicone septum and used for *in vivo* HS-SPME.
216 The vials were cleaned with powered detergent (Alconox, Inc., NY, USA) and were
217 rinsed with hot water and pure water, then dried at 110 ºC overnight prior to use. Empty
218 vials and with five live *H. axyridis* within each vial were weighed by an electronic
219 balance. Each vial with beetles was allowed to equilibrate for 24 h before HS-SPME at
220 30 ºC. Headspace SPME sampling was initiated by piercing the septum with the SPME

221 needle and exposing the SPME fiber to the gases in the vial. Sampling of headspace
222 volatiles with SPME were performed with a manual fiber holder from Supelco
223 (Bellefonte, PA, USA). Headspace samples from live beetles only were considered for
224 analyses, i.e., if the beetles died during sampling, the samples were discarded.
225 Five types of SPME fibers were first examined for MPs extraction efficiency:
226 polydimethylsiloxane (PDMS) 100 µm, 85 µm polyacrylate (PA), 50/30 µm
227 divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS), 65 µm
228 divinylbenzene/polydimethylsiloxane (DVB/PDMS), and 85 µm Carboxen/PDMS
229 (Supelco, Bellefonte, PA). New fibers were used in this study. We counted the
230 extraction-desorption times for each fiber to be less than 40 times maximum for the most
231 used SPME fibers in this study. We did not observe any detrimental effects related to
232 SPME fiber longevity nor we observed any problems with the reproducibility. We
233 routinely check the quality of new (unused fibers) under microscope and return them to
234 manufacturer if there are obvious cracks and flaking that will affect the performance of a
235 SPME fiber. It is critically important to check the performance of the SPME fiber
236 coatings especially when they are being heavily used and perform extractions from liquid
237 phase.
238 Before use, each fiber was conditioned in a heated GC splitless injection port
239 under helium flow according to the manufacturer's instructions. Sampling time for HS-
240 SPME of MP standards was first varied from 1 min to 24 h at 25 ºC to determine the
241 optimal SPME extraction conditions ensuring detection of characteristic odorants. After
242 extraction, the SPME fiber was removed from the vial and immediately inserted into the
243 injection port of GC for analysis. The desorption time of SPME fiber was 40 min at 260

244 ºC. The desorption time was not optimized since it had no effect on the overall
245 throughput of samples analyzed with a fulltime panelist participation.
246
247 *2.5. Direct injection method procedure*
248 An individual standard solution of 1 mg mL^{-1} of each MP was prepared in
249 certified A.C.S.-grade methanol (Fisher Scientific, Pittsburgh, PA) and stored in dark at 4
250 ºC. Stock standard solutions containing all analytes were prepared and subsequently
251 diluted with methanol for six different concentrations (10, 50, 100, 500, 1000, 10000 µg
252 L^{-1}). For direct injections, 1 µL of three MPs standard solution was directly injected into
253 GC-MS under splitless mode condition by using 10 µL gastight syringe (Hamilton, Reno,
254 NV).
255
256 *2.6. Repeatability, reproducibility, and method detection limits*
257 The repeatability was calculated at different levels of concentration: 0.1, 0.5, 5, 10
258 ng L^{-1}, and 0.05 µg L^{-1} ($n = 3$). Data were analyzed and compared using means and
259 relative standard deviation (RSD). The reproducibility was evaluated for 0.1 ng L^{-1} ($n = 3$)
260 by conducting repeated analyses in three different days. The U.S. Environmental
261 Protection Agency (USEPA) methodology for estimation of method detection limits
262 (MDLs) was used [28]. The MDLs were defined as the minimum concentration of a
263 substance that can be measured and reported with 99% confidence when the analyte
264 concentration is greater than zero and is determined from analysis of a sample in a given
265 matrix containing the analyte [28]. The MDLs for MPs was estimated using equation 1:
266 $$MDL = s \times t_{(n-1,\ 1-\alpha=0.99)} \quad (1)$$

267 where:

268 n = number of replicate spike determinations at 1 to 5 times the estimated MDL,

269 s = standard deviation of measured concentrations of n spike determinations,

270 t = Student's t value at n-1 degree of freedom and 1-α (99%) confidence level.

271 When n = 10 and α = 0.01, then t = 2.821, and α = level of significance.

272

273 3. Results and Discussion

274

275 *3.1. Selection of SPME fiber coating*

276 To complete our objective of confirming if MPs are the sole source of noxious

277 odors from *H. axyridis*, we determined which SPME fibers were most sensitive for

278 extracting target analytes (Figure 1). The 50/30 µm DVB/Carboxen/PDMS fiber was the

279 most efficient in extracting target MPs from headspace. Thus, the 50/30 µm

280 DVB/Carboxen/PDMS fiber coating was selected for the subsequent experiments. This

281 finding was consistent with Hartmann et al. [17] and Chapman et al. [29] who used

282 DVB/Carboxen/PDMS for extracting MPs from wine.

283

284 *3.2. Effects of SPME extraction time on MPs and characteristic odors released from live*

285 H. axyridis

286 To determine the relationship between time and MPs extraction we varied

287 exposure periods of SPME fibers to the headspace of five live *H. axyridis* from 1 min to

288 24 h at a constant temperature (30 ºC). Mass selective detector response measured as the

289 mean peak area counts for these four MPs and their single ion integration were used to

290 evaluate the effects of SPME extraction time on those characteristic compounds released
291 by live *H. axyridis* (Figure 2). Abundance of MPs followed a linear trend with increasing
292 SPME extraction time for all compounds tested, especially SBMP and IPMP ($R^2 > 0.99$).
293 Adsorbed amounts of MPs progressively increased with no evidence of reaching sorption
294 equilibrium within the test time range (up to 24 h). There was also an effect of SPME
295 extraction time on odor intensity for the series of MPs tested (Figure 3). In general,
296 longer extraction time resulted in an increase in the odor intensity associated with these
297 characteristic compounds. In all subsequent experiments, extraction time was set at 24 h.
298

299 *3.3. Simultaneous chemical and sensory identification of VOCs released from live* H.
300 axyridis

301 Insect volatiles were analyzed on a multidimensional GC-MS-O system enabling
302 simultaneous identification and analysis of chemicals and corresponding odors.
303 Comparison of a typical total ion chromatogram and aromagram is shown in Figure 4.
304 Thirty eight compounds belonging to 10 chemical groups were identified from the
305 headspace of samples of five live *H. axyridis*, i.e., alkanes and alkenes (4), alcohols (8),
306 aldehydes (3), aromatic hydrocarbons (1), acids (3), halogenated hydrocarbons (1),
307 ketones (9), pyrazines and N-containing compounds (5), S-containing compounds (1) and
308 terpenes (3). A summary of detected compounds and the corresponding odor characters
309 recorded by panelists is provided in Table 2 [30,31]. Thirty two of these compounds were
310 confirmed with pure standards. However, we were unable to locate commercial sources
311 for the remaining six compounds, i.e. 2-ethyl-5-methylthiopene (CAS: 40323-88-4), 3,4-
312 dimethyl-2-hexanol (CAS: 19550-05-1), 2,5-dimethyl-3-methoxypyrazine (CAS: 19846-

313 22-1), dihydro-4-methyl-2[3H]-furanone (CAS:1679-49-8), 5-ethyldihydro-5- methyl-
314 2[3H]-furanone (CAS:2865-82-9) and ionol (CAS:4130-42-1). Therefore their
315 identification is based on high probability matches (>70%) with BenchTop/PBM mass
316 spectral library. An additional eight unidentified compounds with their aroma tags are
317 listed in Table 2, i.e., ’sewer, skunky, fecal’ (aroma peak #1, start time=1.75 min), ’foul’
318 (aroma peak #3, start time =3.13 min), ’sweet’ (aroma peak #5, start time =5.48
319 min), ’mushroom, earthy, moldy, (aroma peak #11, start time=11.95 min), ,’burnt’
320 (aroma peak #18, start time=18.14 min), ’earthy, moldy’ (aroma peak #20, start
321 time=21.20 min), ’moldy, musty’ (aroma peak #22, start time=21.20 min),
322 and ’herbaceous’ (aroma peak #23, start time=21.94 min). These compounds were
323 extracted below the GC-MS detection limit but higher than its odor detection threshold,
324 i.e., their presence was detected by panelists through GC-O and not readily apparent on
325 the resulting total ion chromatogram. This illustrates cases where the use of human nose
326 as a detector in analytical work could be advantageous in finding and identifying of
327 compounds that are ‘overlooked’ and ‘missed’ even with sensitive chemical detectors.
328
329 *3.4. Identification of methoxypyrazines released by live H. axyridis*
330 According to previous studies, it is well known that pyrazines are secreted by
331 lady beetles [2-5]. In this study, the four characteristic odors closely resembling the
332 entire headspace of live beetles were identified as DMMP, IPMP, SBMP and IBMP
333 (Table 2). The familiarity with the overall odor of beetles and their ‘characteristic’ odor
334 was determined in preliminary training of all panelists. We kept live beetles in 40 mL
335 vial for several hours and then opened the cap of the vial, and panelists smelled the odor

336 from the opened vial as part of the training. This organoleptic evaluation of the
337 headspace was continued during the controlled experiments. The only difference was
338 that these evaluations were conducted after SPME extractions from the headspace. The
339 overall odor of live beetles was a mix of 'roasted peanut, nutty, potato, green bell pepper'
340 odor characters.

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

358 SBMP was positively identified by matching the retention time of standard
359 compound, matching mass spectrum of unknown compound with BenchTop/PBM and by
360 matching the odor character, i.e. 'bell pepper', 'peanut', and 'potato'. The extracted
361 amount of IBMP from *H. axyridis* was lower than its identification limit (0.022 ng L^{-1},
362 later discussed in Table 4) based on probability matching. However, IBMP was
363 identified by matching the retention time of standard compound and matching the odor
364 character. One compound was consistently tagged by all panelists with the characteristic
365 odor, i.e., 'roasted peanuts' and later tentatively identified as DMMP with a mass
366 spectrum match greater than 90 % when using the BenchTop/PBM library. Seifert et al.
367 [8] reported 'roasted peanut' aroma and tentatively associated it with methyl MPs without
368 specifically pointing to DMMP. The release of IPMP, SBMP, and IBMP from dead
369 beetles has been reported in previous studies (Table 1) [2,3]. However, we are not aware
370 of any previous report of DMMP released by *H. axyridis*. Because pure DMMP is not
371 commercially available, it could not be confirmed with a standard at this time. However,
372 based on this preliminary chemical and sensory identification, it is important to consider
373 DMMP as another important, fouling odor compounds that is emitted by live *H. axiridis*.
374 Previous studies suggested that IPMP is the most important component of *H.*
375 *axyridis*'s aroma. Cudjoe et al. [3] found IPMP was the most abundant MPs released by
376 dead *Coccinella septempunctata*, *H. axyridis* and *Hippodemia convergens* lady beetles
377 (Coccinellidae). Pickering et al. [10] reported IPMP was detected at relatively high
378 concentration and at levels above sensory threshold in grape juice used for wine
379 fermentation and contaminated with live *H. axyridis*. Pickering et al. [10] also found that
380 IPMP is responsible for the distinctive sensory characteristics of *H. axyridis*

381 contaminated wines and found significant positive correlations between IPMP
382 concentration and specific aroma attributes in wines. Abassi et al. [2] tentatively
383 identified IPMP from dead *C. septempunctata*, and confirmed IPMP is responsible for the
384 characteristic odor of *C. septempunctata* adults (Table 1). The identification was
385 confirmed by peak enhancement on GC with pure standard and by the panelist matching
386 the aroma characteristics. In summary, only tentative identifications of IPMP were
387 achieved using selected ion monitoring of volatiles and comparison of GC retention time
388 in previous studies [2, 3, 6, 10].
389

390 *3.5. Multidimensional GC-MS-O*

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

403 The instrument was first set to GC-FID-O mode with no heart-cut by utilizing the
404 sniff port to identify specific GC pre-column retention times for which eluants exhibit
405 characteristic odor. Figure 5 shows comparison of total ion chromatogram (TIC), FID
406 chromatogram and aromagram of volatiles released by five live *H. axyridis* analyzed in
407 GC-FID-O mode. Based on samples analyzed in GC-FID-O mode, the specific GC pre-
408 column retention times associated with characteristic odors were then selected for
409 activating the multidimensional GC-MS-O mode with the Dean's switch. At first, only
410 three characteristic odors were identified by panelists in the GC-FID-O mode. Due to
411 limited separation capacity of pre-column resulting in two of the MPs coeluting, i.e.
412 SBMP and IBMP, the odor events were merged. When the pre-column heart-cut times
413 were set from 9.00 to 13.00 min and a second replicate was analyzed, only heart-cuts
414 (small segments) of chromatographic effluent were further separated on analytical
415 column and analyzed simultaneously by the MS detector and a panelist at the sniff port.
416 Resulting total ion chromatogram, FID chromatogram and aromagram of heart-cut
417 effluent in MDGC-MS-O mode of volatiles released by *H. axyridis* is shown in Figure 6.
418 The number of aromas was significantly reduced to 7 from 28 in GC-MS-O mode (Figure
419 4). However, IPMP still coeluted with 2-ethyl-1-hexanol. Then, narrower heart-cut times
420 were set from 9.00 to 11.50 min to try to isolate IPMP from 2-ethyl-1-hexanol. As can be
421 seen in Figure 7, the separation of IPMP and 2-ethyl-1-hexanol was much improved even
422 though it was not a baseline separation. Furthermore, the number of aromas was reduced
423 to 2 making it easier for the panelist to record aroma events and then to establish the link
424 with specific chemical. The EI-MS spectrum of IPMP from live *H. axyridis* is shown in
425 Figure 8 (Part A) which was almost identical to that for the standard of IPMP. The match

426 of MS spectrum by BenchTop/PBM was 95%. The MS spectrum for the pure standard
427 IPMP was provided in Figure 8 (Part B) for comparison.
428 The odor character of IPMP, SBMP and IBMP was very similar to that of 'green
429 bell peppers' and 'potatoes'. The odor character of DMMP has been described as 'moldy'
430 and 'earthy' (Table 2) and was consistent to the 'roasted peanut' and 'moldy' character
431 reported by Seifert et al. (1970) [8]. The odor detection threshold of these MPs in air is
432 not known but likely extremely low. Seifert et al. [8] found that the isopropyl and
433 isobutyl methoxypyrazines had an odor detection threshold in the order of 2 ng L^{-1} in
434 water. Seifert et al. [8] reported that shortening the chain from butyl/propyl to ethyl group
435 weakened the odor strength. Further shortening to methyl group resulted in further
436 weakening of odor threshold to approximately 4,000 ng L^{-1} in water [8]. It is therefore
437 reasonable to assume that the MPs discussed in this manuscript have a similar odor
438 detection threshold.
439
440 *3.6. Validation of analytical method*
441 Repeatability and reproducibility of the HS-SPME-GC-MS method were
442 expressed as RSDs of the three MPs. Repeatability was evaluated by analysis of three
443 MPs at seven different concentration levels of 0.1, 0.5, 5 ng L^{-1}, 0.01 and 0.05 μg L^{-1}
444 with three replicates for each concentration. The reproducibility experiment was
445 performed at lowest concentration (0.1 ng L^{-1}) with ten replicates in three different days.
446 The RSDs at different concentrations were less than 8.7% (Table 3). For the 0.1 ng L^{-1}
447 concentration, the intra- and inter-day precision for the three MPs were < 3.9 and 7.8 %.

448 The linearity ranges, linear regression equation and R^2 for HS-SPME-GC-MS
449 method are summarized in Table 4. The calibration curve for three MPs was linear over
450 the concentration range of 0.1 ng L^{-1} -0.05 µg L^{-1}, with R^2 value equal to 0.9984 for
451 IPMP, 0.9976 for SBMP and 0.9958 for IBMP, respectively. Headspace sample recovery
452 was tested using 10 ng L^{-1} standard mixture for three MPs with two consecutive 24 h
453 extractions with HS-SPME. This was done to test the assumption that the MPs are
454 volatized to headspace and that headspace extractions are exhaustive under the optimized
455 SPME extraction conditions. The sample mass recovery was 96% for IPMP, 99% for
456 SBMP and 98% for IBMP, respectively, in the first extraction. The RSDs varied from
457 2.4% to 6.2% (n = 3) for three MPs for the first extraction and from 2.6% to 9.2% (n = 3)
458 for the second extraction.

459 The sensitivity of HS-SPME method was compared with direct injection method.
460 The direct injection method was also used to calibrate MS detector. The linear regression
461 equations and R^2 for direct injection calibration curves were based on six standard
462 concentrations (10, 50, 100, 500, 1000, 10000 µg L^{-1}) and are also presented in Table 4.
463 The R^2 values were 0.9972 for IPMP, 0.9974 for SBMP, and 0.9966 for IBMP,
464 respectively. It is interesting to mention that the difference between the lowest
465 concentrations of standards used for the HS-SPME and direct injection method with
466 comparable MS response was approximately 5 orders of magnitude, i.e., 0.1 ng L^{-1} vs. 10
467 µg L^{-1}.

468 The MDLs for HS-SPME-GC-MS were estimated using equation 1 and are listed
469 in Table 4. The MDLs were estimated and compared for the two approaches used to
470 isolate MPs; (a) HS-SPME-multidimensional-GC-MS, i.e., with heart-cut and (b) HS-

471 SPME-GC-MS without heart-cut. The MDLs were estimated based on the experiment
472 with 10 replicate HS-SPME extractions of the MPs at the lowest concentration (0.1 ng L^{-1})
473 of linearity range using equation 1. The detection limit obtained for HS-SPME-
474 multidimensional-GC-MS method was always lower for each MP and improved by
475 52.2%, 52.4, and 38.9% for IPMP, SBMP, and IBMP, respectively. This improvement is
476 likely due to the reduction of interferences with the introduction of narrow heart-cuts in
477 multidimensional mode.
478

479 3.7. *Estimation of IPMP, SBMP, and IBMP releases per beetle mass and per beetle*

480 The MDGC-MS-O approach was used to quantify MPs released to headspace
481 using SPME and *in vivo* sampling. The estimated amounts of three MPs emitted from live
482 *H. axyridis* are presented in Table 5. The average amounts of three MPs per beetle mass
483 (for n = 8 replicates for red beetles, with each replicate comprised of five beetles in a 40
484 mL vial) were 8.0569 ng g^{-1} for IPMP, 3.1680 ng g^{-1} for SBMP and 0.0811 ng g^{-1} for
485 IBMP, respectively. The average amounts of three MPs per beetle mass (for n = 2
486 replicates for orange beetles, with each replicates comprised of five orange beetles in a 40
487 mL vial) were 0.4111 ng g^{-1} for IPMP, 0.6191 ng g^{-1} for SBMP and 0.0055 ng g^{-1} for
488 IBMP, respectively. For pooled red and orange beetles, the average were 4.2340 ng g^{-1}
489 for IPMP, 1.8965 ng g^{-1} for SBMP and 0.0091 ng g^{-1} for IBMP, respectively. The
490 amounts of MPs estimated as mass per beetle (Table 5) are consistent with the odor
491 intensity recorded by three panelists. IPMP had the strongest odor and was the most
492 abundant MP (Table 5). The dominance of IPMP among other MPs emitted from *H.*

493 *axyridis* is consistent with observations by Cudjoe et al. [3] who reported that IPMP had
494 the greatest aroma intensity due to its higher gas emissions from frozen/thawed beetles.
495 Although it was not our goal to determine the relationship between adult color
496 and MP concentrations, we did observe evidence of such a relationship (Table 5). There
497 is a great variation in the color and marking patterns of *H. axyridis* [32]. In North
498 America, adults vary from a bright red to a pale orange color with a range in the number
499 and location of black spots. Outside of North America, phenotypic variation is even
500 greater including black morphs with orange to red spots. This aposematic coloration is
501 determined both by genetic factors and the influence of the larval diet [33]. Although the
502 relationship is not clear between color intensity of adult *H. axyridis* and MP content [34],
503 we separated beetles by apparent color to reduce a possible source of variation.
504 Interestingly, the average concentration of all MPs (IPMP, SBMP, and IBMP) were
505 lowest in the orange replicates and the highest in the red replicates (mean $\pm$ standard
506 deviation; 1.036 $\pm$ 0.46 ng g^{-1} and 11.243 $\pm$ 10.32 ng g^{-1} for orange and red beetles,
507 respectively). Although this represents a small, unbalanced data set, we did observe a
508 significant difference between these two means (t = 2.89, df =9, P = 0.02; PROC TTEST,
509 SAS 2002) [35]. If there is a relationship between adult color and MP concentrations,
510 this would help explain differences in our estimates of *H. axyridis* MP concentrations
511 versus those of other investigators that did not account for this potential source of
512 variation [3].
513 The estimated emissions of three MPs were also compared for the two
514 methodologies (direct injection vs. HS-SPME; Figure 9). Ten vials with five live beetles
515 for each vial were analyzed by both methods. The individual data, correlation and

516 regression analyses are presented in Figure 9 (Part A, B and C). All correlation

517 coefficients were greater than 0.99, indicating good linearity, i.e., for IPMP (R^2=0.9999)

518 and SBMP (R^2=0.9976). For IBMP, linear relationship was less robust (R^2=0.7174, data

519 not shown in Figure 9). This is likely due to the uncertainties associated with measured

520 low concentrations near the estimated MDL. However, if the calibration curve for IBMP

521 is plotted through the origin, there was a linear relationship for IBMP (R^2=1.0000)

522 between two methods (direct injection vs. HS-SPME). The slopes of regression line for

523 three MPs were greater than 0.82 indicating that both methods were in good agreement.

524

525 4. Conclusions

526 *In vivo* HS-SPME combined with multidimensional GC-MS-O has a great

527 potential for investigating links between specific chemicals released by insects and their

528 characteristic odors. In this research, 50/30 µm DVB/Carboxen/PDMS SPME fiber was

529 used to extract headspace volatiles released by live *H. axyridis*. Thirty eight compounds

530 were identified in headspace of live *H. axyridis* including four characteristic odorous

531 compounds-DMMP, IPMP, SBMP and IBMP. We detected a previously unidentified MP

532 (DMMP) that appears to be also a component of *H. axyridis*'s odor. We also provided

533 conclusive evidence that IPMP released within the headspace of living *H. axyridis* is

534 responsible for their characteristic odor. Quantification of three MPs (IPMP, SBMP and

535 IBMP) emitted from live beetles was performed using external calibration curves by HS-

536 SPME-MDGC-MS. Linear relationships (R^2 was > 0.9958 for all 3 MPs) were observed

537 over a concentration range from 0.1 ng L^{-1} to 0.05 µg L^{-1}. The MDLs were estimated at

538 0.022 ng L^{-1}, 0.020 ng L^{-1}, 0.022 ng L^{-1} for IPMP, SBMP, and IBMP, respectively.

539 These MDLs obtained with multidimensional GC-MS with heart-cut approach represent
540 52.2%, 52.4%, and 38.9% improvement compared to GC-MS without heart-cut approach.
541 For the 0.1 ng L^{-1} concentration, the intra- and inter-day precision for the three MPs were
542 less than 3.9 and 7.8 %. Using the HS-SPME-MDGC-MS method we estimated that live
543 *H. axyridis* per beetle body mass released 4.2340 ng g^{-1} of IPMP, 1.8965 ng g^{-1} of SBMP
544 and 0.0091 ng g^{-1} of IBMP. We observed a significant correlation between MP emissions
545 and beetle color, with orange beetles releasing more MP than yellow beetles. Although
546 this observation requires further experiments with a more balanced sample, it represents a
547 previously undocumented source of variability in MP from *H. axyridis*. We conclude that
548 HS-SPME-MDGC-MS-O is a novel, fast and reliable methodology for the determination
549 of characteristic odorants (MPs) emitted from live *H. axyridis*. This method has potential
550 to be used for rapid *in vivo* determination of odor-causing chemicals associated with
551 emissions of volatiles from other insects.

552 **Acknowledgments**

553 The authors would like thank Iowa State University for funding this study.

554 **References**

555
556 [1]. M. Huelsman, J. Kovach, American Entomologist, 50 (2004) 16.
557 [2]. S. Al Abassi, M.A. Birkett, J. Pettersson, J.A. Pickett, C.M. Woodcock, Cell. Mol.
558 Life Sci., 54 (1998) 876.
559 [3]. E. Cudjoe, T.B. Wiederkehr, I.D. Brindle, Analyst, 130 (2005) 152.
560 [4]. A.G. King, J. Meinwald, Chem. Rev. 96 (1996) 1105.
561 [5]. J.R. Aldrich, W.S. Leal, R. Nishida, A.P. Khrimian, C.J. Lee, Y. Sakuratani,
562 Entomologia Experimentalis et Applicata, 84 (1997) 127.

563 [6]. B.P. Moore, W.V. Brown, M. Rothschild, Chemoecology, 1 (1990) 43.

564 [7]. R.G. Buttery, R.M. Seifert, D.G. Guadagni, L.C. Ling, J. Agric. Food Chem. 17
565 (1969) 1322.

566 [8]. R.M. Seifert, R.G. Buttery, D.G. Guadagni, D.R. Black, J.G. Harris, J. Agric.
567 Food Chem. 18 (1970) 246.

568 [9]. M.S. Allen, M.J. Lacey, In Waterhouse, A. L.; Ebeler, S. E., Eds. Chemistry of
569 wine flavor. American Chemical Society, NY, 1998, p. 31.

570 [10]. G. Pickering, Y. Lin, A. Reynolds, G. Soleas, R. Riesen, I. Brinndle, J. Food Sci.,
571 70 (2005) s128.

572 [11]. G. Pickering, J. Lin, R. Riesen, A. Reynolds, I. Brindle, G. Soleas, Am. J. Enol.
573 Vit. 55 (2004) 153.

574 [12]. G. Pickering, J. Lin, A. Reynolds, G. Soleas, R. Riesen, Int. J. Food Sci. Technol.,
575 41 (2006) 77.

576 [13]. D. Ryan, P. Watkins, J. Smith, M. Allen, P. Marriott, J. Sep. Sci., 28 (2005)1075.

577 [14]. K.M. Girsch, R.E. Foster, Proceedings of the Annual Meeting of The
578 Entomological Society of America, Fort Lauderdale, FL, 2005.

579 [15]. A. Zhang, J.E. Oliver, J. R. Aldrich, B. Wang, V.C. Mastro, Anoplophora
580 glabripennis (Motschulsky). Z. Naturforsch. 57c (2002) 553.

581 [16]. M.J. Lacey, M.S. Allen, R.L.N. Harris, W.V. Brown, Am. J. Enol. Vitic. 42 (1991)
582 103.

583 [17]. P.J. Hartmann, H.M. McNair, B.W. Zoecklein, Am. J. Enol. Vitic. 53 (2002) 285.

584 [18]. C. Prouteau, R. Schneider, Y. Lucchese, F. Nepveu, R. Renard, C. Vaca-Garcia,
585 Anal. Chim. Acta, 513 (2004) 223.

586 [19]. C. Sala, M. Mestres, M.P. Marti, O. Busto, J. Guasch, J. Chromatogr. A. 880
587 (2000) 93.
588 [20]. C. Sala, M. Mestres, M.P. Marti, O. Busto, J. Guasch, J. Chromatogr. A 953
589 (2002) 1.
590 [21]. J.A. Koziel, J. Pawliszyn, J. Air Waste Manage. Assoc. 51 (2001) 173.
591 [22]. F. Augusto, A.L.E. Lopes, C.A. Zini, TrAC, Trends Anal. Chem. 22 (2003) 160.
592 [23]. D.W. Wright, L. Nielsen, D. Eaton, F. Kuhrt, J.A. Koziel, J.P. Spinhirne, D.B.
593 Parker, J. Agric. Food Chem. 53 (2005) 8663.
594 [24]. D.W. Wright, In: Techniques for Analyzing Food Aroma. Marsili R., Ed. Marcel
595 Dekker, Inc., NY, 1997; p 113.
596 [25]. E.A. Bulliner IV, J.A. Koziel, L. Cai, D. Wright, J. Air Waste Manage. Assoc. 56
597 (2006) 1391.
598 [26]. L. Cai, J.A. Koziel, J. Davis, Y.C. Lo, H. Xin, Anal. Bioanal. Chem. 386(2006)
599 1791.
600 [27]. A.H. Heymann, A.C. Noble, R.B. Boulton, J. Agric. Food. Chem. 34 (1986) 268.
601 [28]. http://www.dnr.wisconsin.gov/org/es/science/lc/OUTREACH/-
602 Publications/LOD%20Guidance%20Document.pdf, accessed on Nov. 7, 2006.
603 [29]. D.M. Chapman, J.H. Torngate, M.A. Matthews, J. Guinard, S.E. Ebeler, J. Agric.
604 Food. Chem. 52 (2004) 5431.
605 [30]. M. Devos, F. Patte, J. Roualt, P. Laffort, L.J. Van Gemert, Standardized Human
606 Olfactory Thresholds, IRL Press at Oxford Press, NY, New York, 1990.
607 [31]. http://www.flavornet.org/flavornet.html, Accessed on Nov. 10, 2006.
608 [32]. R. L. Koch, J. Insect Sci. 3 (2003) 32. www.insectscience/org/3.32

609 [33]. R.F. Preziosi, W. E. Snyder, C. P. Grill, A. J. Moore, Evol. 53 (1999) 1312

610 [34]. C.P. Grill, Evol. Ecol. Res. 1(1999) 651.

611 [35]. SAS Institute Inc., 2002. SAS OnlineDoc, Version 9.1 SAS Institute Inc. Cary,

612 NC

613

614

615 # Figure captions

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

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

628 **Figure 3.** Effect of time on HS-SPME extraction of the odor intensity of four
629 characteristic odors released by live *H. axyridis* with 50/30 µm
630 DVB/Carboxen/PDMS fiber. Extraction time = 1 min, 15 min, 1 h, 6 h, 12 h and
631 24 h. Error bars show the standard deviation of the mean (n=3).

632 **Figure 4.** Comparison of total ion chromatogram and aromagram with full heart-cut
633 mode of headspace gases released by live *H. axyridis* in September (2005) and
634 collected with 50/30 µm DVB/Carboxen/PDMS SPME using 24 h sampling
635 time. Identified compounds, odors and aromas are summarized in Table 2.
636 DMMP = 2, 5-dimethyl-3-methoxypyrazine; IPMP = 2-isopropyl-3-
637 methoxypyrazine; SBMP = 2-sec-butyl-3-methoxypyrazine; IBMP = 2-isobutyl-

638 3-methoxypyrazine. Arrow marks odorous 2-ethyl-1-hexanol co-eluting with
639 IPMP in this GC-MS-O mode.
640 **Figure 5.** Separations in GC-FID-O mode with no heart-cut: comparison of
641 Chromatogram (FID) and aromagram for the headspace of 5 live *H. axyridis*
642 and collected with 50/30 µm DVB/Carboxen/PDMS SPME using 24 h sampling
643 time, extraction temperature = 30 ºC
644 **Figure 6.** Separations for MPs from the headspace of 5 live *H. axyridis* in MDGC-MS-O
645 mode with heart-cut between pre-column and analytical column: comparison of
646 the FID chromatogram, total ion chromatogram and aromagram isolating only
647 characteristic odorants. Heart-cut time range: 9.00-13.00 min.
648 **Figure 7.** Separations for MPs from the headspace of 5 live *H. axyridis* in MDGC-MS-O
649 mode with heart-cut between pre-column and analytical column: comparison of
650 the FID chromatogram, total ion chromatogram and aromagram isolating only
651 characteristic odorants. Narrower heart-cut time range: 9.00-11.50 min was
652 used to isolate aromas caused by IPMP and 2-ethyl-1-hexanol.
653 **Figure 8.** The electron impact-mass spectrum (EI-MS) of IPMP isolated from volatiles
654 collected from headspace of five live *H. axyridis* (Part A) and pure standard
655 (Part B). MS scan mode: total ion scan mode.
656 **Figure 9.** Comparison of MPs emissions from live *H. axiridis* to headspace estimated
657 with two different methods of calibration: HS-SPME-GC-MS with heart-cut
658 and by direct injection in GC-MS with heart-cut. Part A: IPMP; Part B: SBMP;
659 Part C: IBMP. MS scan mode: SIM mode.* All measured concentrations of
660 MPs from live *H. axiridis*

661 were above the estimated MDL and below the lowest linear concentration;

662 calibration curve for IBMP was forced through origin.