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Determination of 3-Alkyl-2-methoxypyrazines in Lady Beetle-Infested Wine by Solid-Phase Microextraction Headspace Sampling

TEDERSON LUIZ GALVAN,* STEPHEN KELLS, AND WILLIAM DALE HUTCHISON

Department of Entomology, 219 Hodson Hall, 1980 Folwell Avenue, University of Minnesota, St. Paul, Minnesota 55108

This study determined the concentration of 3-alkyl-2-methoxypyrazines in Frontenac and Leon Millot wines made from grapes that were naturally or artificially infested with the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). Headspace sampling with solid-phase microextraction (SPME) and gas chromatography (GC) was used for the quantification of 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP), and 3-isobutyl-2-methoxypyrazine (IBMP). The resulting method parameters included linearity ($r^2 > 0.98$), limit of detection (>0.25 ng/L), relative standard deviation (<20%), and recovery (75–125%). IPMP concentrations in wine were not significantly different among the levels of natural or artificial infestations of *H. axyridis*. SBMP was found only in wine artificially infested with *H. axyridis*. IBMP was found in wine artificially infested with *H. axyridis* and in Frontenac wine, but not in Leon Millot. The consequences of these results for future research in the contamination of wine with *H. axyridis* are discussed.

KEYWORDS: *Harmonia axyridis*; headspace-SPME; wine grapes; pyrazines; contaminant pest

The multicolored Asian lady beetle, *Harmonia axyridis* (Pallas), has become an economically significant contaminant pest in wine production throughout the Great Lakes region in North America. Adults aggregate on clusters containing injured berries just prior to harvest (*1–3*), and once disturbed or crushed these beetles release a yellow fluid from the tibio-femoral joints of their legs (*4, 5*). The process of fluid release is often referred to as "reflex bleeding" (*4, 6*). The fluid contains alkaloids used for defense and 3-alkyl-2-methoxypyrazines that could be used as an aggregation pheromone or in Müllerian mimicry due to their strong smell (*4–7*). One 3-alkyl-2-methoxypyrazine, the 3-isopropyl-2-methoxypyrazine (IPMP), has been suggested to be one of the key compounds responsible for the off-flavor produced by *H. axyridis* in wines (*8*). In addition to IPMP, two other 3-alkyl-2-methoxypyrazines, 3-*sec*-butyl-2-methoxypyrazine (SBMP) and 3-isobutyl-2-methoxypyrazine (IBMP), could also be affecting the wine flavor because they are found in *H. axyridis* (*7, 9*). These compounds are also well-known for their contribution to vegetative, herbaceous, green bell pepper, and earthy character of wines such as Cabernet Sauvignon and Sauvignon Blanc (*10, 11*).

Pyrazines, including the 3-alkyl-2-methoxypyrazines, are odiferous compounds that can be noticed by humans even at minute concentrations such as a few parts per trillion (ppt) or nanograms per liter (ng/L). The sensory threshold of IBMP in water is between 1 and 2 ng/L (*12, 13*). In wine, the sensory thresholds of 3-alkyl-2-methoxypyrazines were determined for several varieties that contain natural levels of these compounds. For example, the sensory threshold of IBMP in Sauvignon Blanc was 8 ng/L (*14*), and that in Cabernet Sauvignon, Cabernet Franc, and Merlot was 15 ng/L (*15*). These thresholds were determined using gas chromatography−mass spectrometry (GC-MS) techniques and tasting panels.

In red and white wines, IPMP has been shown to affect sensory properties such as bell pepper and peanut aromas (*8*). However, the quantification of IPMP, SBMP, and IBMP from *H. axyridis* infestation levels in wine grapes of Frontenac has not been determined. Evidence that 3-alkyl-2-methoxypyrazines are indeed associated with the characteristic off-flavor from *H. axyridis* in wine and the quantification of these compounds are important steps toward the development of remediation procedures to control the lady beetle taint. A pest management program for this insect is being developed, and includes the use of practical sampling plans (*3*), action thresholds (*16*), and chemical control (*17*). However, remediation of *H. axyridis* contamination during wine processing is essential for grape growers averse to insecticide use, and winemakers that do not have the options for controlling lady beetle populations in the vineyard.

In this paper, we determined the concentration of 3-alkyl-2-methoxypyrazines in Frontenac wine from grapes, artificially or naturally infested with *H. axyridis*, using headspace sampling with solid-phase microextraction (SPME) fibers and gas chromatography (GC). Frontenac is a red grape variety that has become one of the most important for cold climates worldwide and is helping to expand the wine grape industry to unusual

* Author to whom correspondence should be addressed [telephone (612) 624-3670; fax (612) 625-5299; e-mail galva008@umn.edu.

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grape-growing regions such as Minnesota (*18*). In addition, we also determined the concentrations of IPMP, SBMP, and IBMP in Leon Millot wine.

MATERIALS AND METHODS

Insects. *H. axyridis* adults were collected during August 2005 in soybean fields at the Rosemount Research and Outreach Center, University of Minnesota, Rosemount, MN. Adults were identified using a diagnostic guide (*19*), and voucher specimens were deposited in the University of Minnesota Insect Museum (Department of Entomology, University of Minnesota). Following collection, adults were held in 1.96 L plastic dishes with ~100 beetles per dish and maintained at 10 ± 1 °C with a photoperiod of 16:8 (L:D) h. Three days prior to use, the dishes containing *H. axyridis* were warmed to 25 ± 1 °C with a photoperiod of 16:8 (L:D) h. *H. axyridis* adults were provided an ad libitum supply of live soybean aphids, *Aphis glycines* Matsumara, pea aphids, *Acyrthosiphon pisum* (Harris), a diet made from freeze-dried drone honey bee, *Apis mellifera* L., pupae (*20*), and water in cotton balls placed in plastic Petri dishes (60 mm × 15 mm).

Frontenac Grapes. In September 2005, 100 kg of Frontenac wine grapes was harvested from a commercial vineyard thought to be free from *H. axyridis* in Lake City, MN. The grapes were hand picked and visually inspected to ensure no *H. axyridis* were present. Following harvest, and after inspection, 7 kg of grapes was placed into a 22 L polycarbonate container and transported to the Enology Laboratory at the Horticultural Research Center, University of Minnesota, Chaska, MN.

Infestation of Frontenac Grapes. Artificial infestations of the grapes with *H. axyridis* were prepared by adding 1, 3, and 8 live beetles per kilogram of grapes to 22 L polycarbonate containers. Three 22 L polycarbonate container replicates of each treatment were prepared. The containers were closed, inverted, and rolled for 45 s to mimic the disturbance that might be expected during the grape harvest. Grapes with *H. axyridis* adults were then processed and winemaking commenced using standard microvinification techniques. Wine was processed separately for each replicate. During the racking process *H. axyridis* were removed from the treatments (*8*).

Leon Millot Grapes. In September 2004, 14 kg of Leon Millot wine grapes was harvested from a commercial vineyard in Hastings, MN. Grapes were harvested from two plots. In one plot, 20% of clusters were estimated to be infested with one or more *H. axyridis* adults. In the other, 50% of clusters were estimated to be infested with one or more *H. axyridis* adults. Infestation levels were estimated by sampling 60 clusters from each plot at harvest. The plot with 20% of clusters infested with *H. axyridis* was covered with a floating row cover (FRC) for the 2 weeks preceding the harvest. The FRC covered the entire foliage and clusters of the vines, and it was fastened with staples 0.30 m above the ground. The plot with 50% of clusters infested with *H. axyridis* was an untreated plot. Grapes were hand picked, and 7 kg of grapes was placed into a 22 L polycarbonate container and transported to the Enology Laboratory at the Horticultural Research Center, University of Minnesota, Chaska, MN. At the Enology Laboratory, all clusters were carefully hand sorted, and any *H. axyridis* or other lady beetles were removed.

Wine Processing. Wine from Leon Millot (2004) and Frontenac (2005) grapes were prepared using microvinification procedures in 2004 and 2005, respectively. Crushing and destemming was done mechanically in each 22 L polycarbonate container. All wine batches were inoculated with Pasteur Red yeast (Red Star, Milwaukee, WI) at a rate of 265 mg/L. Total fermentation was conducted at 21 °C in containers that were approximately two-thirds full. After 5 days, wine was racked to glass carboys to finish fermentation. Malolactic culture (Chris Hansen CH 35) was added toward the end of fermentation to reduce acidity. After malolactic fermentation completion, sulfur dioxide was added at a rate of 50 mg/L. Wine batches were racked to full containers and cold stabilized for 2 weeks. Each replicate was assessed for pH using an Accumet pH-meter (Fisher Scientific, Pittsburgh, PA), sugar content using a refractometer, and titratable acidity using standard NaOH titration. Finally, the wine was bottled in standard 350 mL splits with natural corks and stored at 21 °C.

Sample Preparation for SPME. The following pure standards were purchased from Pyrazine Specialties (Atlanta, GA): 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP), 3-isobutyl-2-methoxypyrazine (IBMP), and the internal standard (IS) 3-ethyl-2-ethoxypyrazine (EEP). Pure standards were used to develop calibration curves (0, 5, 10, 25, 50, and 100 ng/L of IPMP, SBMP, and IBMP) and to identify the 3-alkyl-2-methoxypyrazines from wine solutions. Samples were prepared using 60% model wine and 40% wine. Model wine was prepared using an ultrapure, type I water source (Milli-Q Water Systems, Billerica, MA) with 0, 12, or 42% (v/v) ethanol and 2 g/L potassium bitartrate. The final ethanol concentrations of samples combining model wine and wine were 5, 12, and 30% (v/v). Ethanol concentration for the wine treatments was determined by ebulliometry. The model wine pH averaged 3.3.

Samples (12 mL) were placed in 20 mL round-bottom glass headspace vials with 3.6 g of sodium chloride, a magnetic stirring bar, and 10 μL of 120 μg/L EEP in ethanol for a final internal standard concentration of 100 ng/L. Sample vials were immediately sealed with magnetic metal crimp tops with silicone/PTFE septum, shaken for 10 s, and stored overnight in the dark at room temperature. For every sample, three vials were prepared.

Two SPME fibers were used: StableFlex 85 μm Carboxen/polydimethylsiloxane (CAR/PDMS) (57299-U) and StableFlex 50/30 μm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (57328-U) (Supelco, Bellefonte, PA). On each sampling day, fibers were conditioned before use according to the manufacturer's instruction (85-μm CAR/PDMS for 1.5 h at 300 °C; 50/30 μm DVB/CAR/PDMS for 1 h at 270 °C). The SPME fiber was then inserted into the headspace of prewarmed (30 min at 40 °C) sample vials for extraction. Temperature (40 °C) and agitation were constant throughout the extraction periods (30, 90, 120, 150, and 180 min). After extraction, the SPME fiber was removed and introduced for 10 min into the injector port of the gas chromatograph. Each vial was sampled only once.

Gas Chromatography (GC). Sample analyses were performed with an Agilent 6890 GC (Palo Alto, CA) equipped with a nitrogen phosphorus detector (NPD). NPD temperature was set at 325 °C, at a constant flow mode with hydrogen flow at 3 mL/min, air flow at 60 mL/min, and makeup flow at 10 mL/min. The splitless GC injector was maintained at 260 °C. The fiber was desorbed for 5 min, and an additional 5 min was used to clean the fiber to a purge flow of 50 mL/min. The purge time was sufficient to not interfere with concurrent samples. Samples were separated on Agilent DB Wax column (30 m × 0.25 mm × 0.25 μm) at a constant flow (0.9 mL/min) and initial pressure of 8.38 psi. Oven temperature was programmed as follows: 35 °C and held for 1 min, 25 °C/min to 100 °C and held for 15 min, 25 °C/min to 230 °C and held for 7 min.

Statistical Analysis. Means and relative standard deviations (RSD) were estimated from three to four replicates. Protected least significant difference test (LSD) and Student's *t* test were used to compare treatments using SAS [PROC ANOVA and PROC TTEST; (*21*)]. Linear regressions and analysis of variance were performed using SAS [PROC GLM; (*21*)].

RESULTS AND DISCUSSION

Fiber Selection. The DVB/CAR/PDMS fiber showed good linearity ($r^2_{IPMP} = 0.94$, $r^2_{SBMP} = 0.96$, $r^2_{IBMP} = 0.95$) for the 3-alkyl-2-methoxypyrazines in a solution of 40% Frontenac wine and 60% wine model. This type of SPME fiber is common in the quantification of semivolatile compounds such as 3-alkyl-2-methoxypyrazines in wine and insects (*9, 22, 23*). However, under our experimental conditions, DVB/CAR/PDMS fibers showed high variability (RSD_{IPMP} = 11–62%, RSD_{SBMP} = 4–66%, and RSD_{IBMP} = 3–53%). The second fiber that we tested, CAR/PDMS, showed better linearity (**Table 1**) and less variability (**Table 2**) than the DVB/CAR/PDMS fiber. CAR/PDMS fibers have had high recoveries of 3-alkyl-2-methoxypyrazines in model wine when used with the NPD detector and 5% phenyl-dimethylpolysiloxane column (*24*). However, these high recoveries were accompanied with distorted and broad GC

Table 1. Quantification Parameters Using CAR/PDMS Fiber: Regression Line, Standard Deviation (SD), 95% Confidence Limits (CL) for the Slope and Intercept, and Limit of Detection (LOD) for Each Analyte

analyte	av regression line[a]	r^2	SD	95% CL (slope)	95% CL (intercept)	LOD[b] (ng/L)
IPMP	$(A_{IPMP}/A_{IS}) = 0.558$ [IPMP/100] (ng/L) + 0.039	0.9887	0.0254	0.4753–0.6414	0.0003–0.0783	0.29
SBMP	$(A_{IPMP}/A_{IS}) = 1.184$ [SBMP/100] (ng/L)	0.9976	0.0248	1.1031–1.2622	−0.025–0.0511	0.25
IBMP	$(A_{IPMP}/A_{IS}) = 1.502$ [IBMP/100] (ng/L) + 0.095	0.9967	0.0369	1.3809–1.6224	0.0379–0.1514	0.46

[a] Four replications of each standard solution concentration (0, 5, 10, 25, 50, and 100 ng/L). A_{IS} = internal standard (EEP at 100 ng/L). [b] LOD = intercept + 10SD for each analyte (*26*).

Table 2. Repeatability Test for *Harmonia axyridis*-free Frontenac Wine Spiked with 3-Alkyl-2-methoxypyrazines Using CAR/PDMS Fiber

analyte added (ng/L)	measured mean ($n = 3$) (ng/L)	lower measurement (ng/L)	upper measurement (ng/L)	SD[a] (ng/L)	RSD[a] (%)
		IPMP			
10	11.54	11.42	11.69	0.13	1.1
25	20.57	18.62	21.93	1.74	8.4
50	54.93	50.65	58.30	3.91	7.1
100	99.12	85.56	118.65	17.33	17.5
		SBMP			
10	12.50	10.84	14.00	1.59	12.7
25	22.44	20.99	24.12	1.58	7.0
50	51.01	46.87	56.37	4.87	9.5
100	100.96	89.67	117.44	14.59	14.4
		IBMP			
10	8.54	7.07	10.42	1.71	20.0
25	18.88	17.52	19.76	1.20	6.3
50	45.42	44.61	46.50	0.97	2.1
100	98.97	89.73	113.43	12.68	12.8

[a] SD = standard deviation; RSD = relative standard deviation (SD × 100/mean).

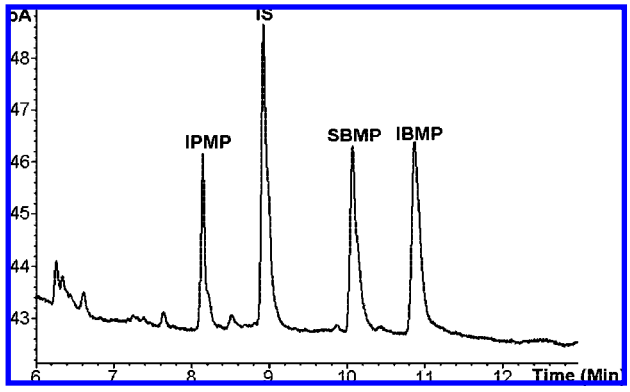

Figure 1. Chromatogram of a Frontenac wine sample analyzed using headspace-SPME/GC procedures. The sample was spiked with 50 ng/L of 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP), 3-isobutyl-2-methoxypyrazine (IBMP), and 100 ng/L of 3-ethyl-2-ethoxypyrazine as the internal standard (IS).

peak areas with considerable tailing (*24*). In our study, we also used the NPD system, but the GC was equipped with a DB-Wax column resulting in more acceptable peaks for chromatograms (**Figure 1**). Therefore, CAR/PDMS fibers were selected for final determination of 3-alkyl-2-methoxypyrazines in our experiment.

Extraction Time. Extraction by headspace-SPME via CAR/PDMS fiber is optimized by reaching equilibrium between the liquid phase and headspace and between the headspace and fiber. The equilibrium among the liquid phase (wine model and wine solution), headspace of the sample vial, and CAR/PDMS fiber for IPMP, SBMP, and IBMP was achieved at 150 min of fiber exposure into the headspace (**Figure 2**). The extraction time of

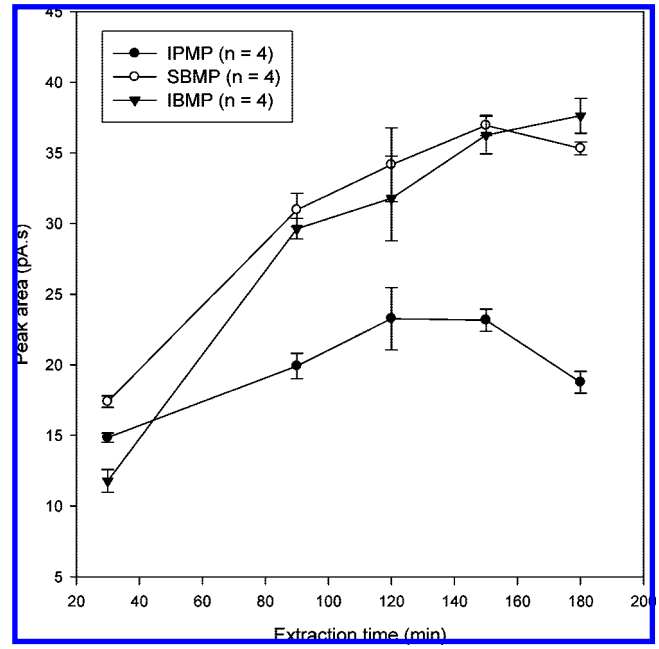

Figure 2. Effect of extraction time on mean peak area (pA.s) of 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP), and 3-isobutyl-2-methoxypyrazine (IBMP) using headspace-SPME/GC procedures.

3-alkyl-2-methoxypyrazines from wine using SPME has ranged from 30 min with DVB/CAR/PDMS fibers (*22*) to 4 h with polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers (*11*). Differences in the extraction times of these compounds from wine are due to experimental conditions such as fiber type, ethanol concentration, temperature of extraction, and GC conditions (*25*).

Ethanol Concentration. A decrease in ethanol concentration from 30 to 5% (v/v) considerably increased 3-alkyl-2-methoxypyrazine recoveries (**Figure 3**). The influence of ethanol in the extraction of these compounds from wine using headspace-SPME fibers is well-known (*11, 25*), and several methods (e.g., distillation of wine) have been used to decrease ethanol concentration before extraction by SPME (*11*). In the current study, we decreased ethanol content in the samples by mixing wine (40%) with model wine (60%) at 0, 12, and 42% (v/v), making a final solution with 5, 12, or 30% (v/v) of ethanol. Because higher recoveries resulted from the solution with 5% (v/v), the determination of 3-alkyl-2-methoxypyrazines in wine was performed using ethanol-free model wine.

Linearity. Three standard curves were developed using a solution of 40% Frontenac wine made from *H. axyridis*-free grapes and 60% model wine (ultrapure, type I water source, 2 g/L potassium bitartrate, no ethanol, and pH 3.3), CAR/PDMS fiber, 150 min of fiber exposure into the headspace, and a solution with 5% (v/v). Four replications were run of each standard concentration (0, 5, 10, 25, 50, and 100 ng/L of IPMP, SBMP, and IBMP) with 100 ng/L of EEP as the internal

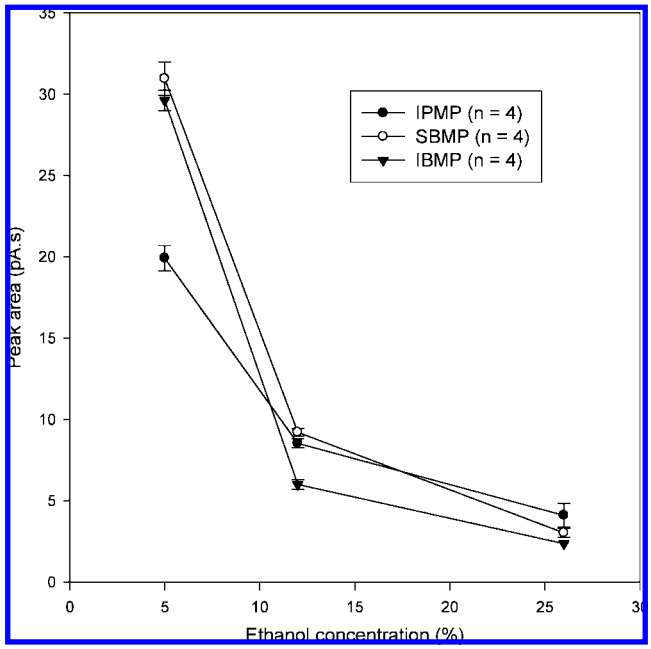

Figure 3. Effect of ethanol concentration on peak area (pA.s) of 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP), and 3-isobutyl-2-methoxypyrazine (IBMP) using headspace-SPME/GC procedures.

standard. All three calibration curves linearly correlated the GC peak curves with the 3-alkyl-2-methoxypyrazine concentrations (**Table 1**).

Limit of Detection (LOD). In this study, LOD was defined as the compound concentration that results in a signal equal to the blank signal (intercept) plus 10 standard deviations (*26*). The LOD was below 1 ng/L for all compounds tested in this study (**Table 1**). Because the sensory threshold of 3-alkyl-2-methoxypyrazines by humans is between 1 and 2 ng/L in water (*12, 13*) and between 8 and 15 ng/L in wine (*14, 15*), the proposed method is adequate for quantification of these compounds in wines using headspace-SPME-GC procedures.

Repeatability and Recovery. The precision of the headspace-SPME-GC method was estimated by the repeatability test of four concentrations (10, 25, 50, and 100 ng/L) for IPMP, SBMP, and IBMP in *H. axyridis*-free Frontenac wine (**Table 2**). The relative standard deviation (RSD) was below 20% for all 12 analyte−concentration combinations. The accuracy of the proposed procedures was calculated by the recovery of IPMP, SBMP, and IBMP spiked into *H. axyridis*-free Frontenac wine at four concentrations (10, 25, 50, and 100 ng/L) (**Table 3**). For the three compounds, recoveries were closer to 100% (i.e., 100% of accuracy) at 50 and 100 ng/L. Therefore, the precision and accuracy of our method to quantify 3-alkyl-2-methoxypyrazines in wine are as good as those of previous methods using headspace extraction by SPME (*11, 22, 27*).

Wine Treatments. Sugar content (12.20−12.70%), pH (3.48−3.58), and titratable acidity (7.06−7.53) did not differ among treatments. Quantification of 3-alkyl-2-methoxypyrazines in Frontenac wine from grapes with increasing levels of *H. axyridis* infestation showed no significant differences in the concentration of IPMP among treatments ($p = 0.13$) (**Table 4**). The concentrations of SBMP ($p < 0.01$) and IBMP ($p < 0.01$) in the treatment infested with 1 *H. axyridis*/kg of grapes was higher than that of other treatments (**Table 4**). In a previous study, where the same treatments were included in a tasting panel to determine the sensory threshold of *H. axyridis* in Frontenac wine, panelists found no significant differences in taste and flavor between the wine infested with 1 *H. axyridis*/kg of grapes and the untreated wine (*16*). However, panelists noticed significant differences between wine infested with either 3 or 8 *H. axyridis*/kg of grapes and the untreated wine (*16*). Comparison between the concentration of 3-alkyl-2-methoxypyrazines in the current study and the tasting panel results does not support the hypothesis that IPMP, SBMP, IBMP, or the combination of these three compounds, would account for the *H. axyridis*-related taint in infested wine (*3, 7, 8*). However, our results do not exclude the possibility that IPMP, SBMP, and IBMP, either isolated or in combination, may contribute along with other factors to the *H. axyridis*-related off-flavor in wine.

We also quantified IPMP, SBMP, and IBMP in Leon Millot wine from grapes with 20 and 50% of clusters infested with at least one *H. axyridis* adult (**Table 5**). In both treatments, concentrations of IPMP were similar ($p = 0.62$), and SBMP and IBMP concentrations were below quantification limits (**Table 5**). However, the tasting panel observed differences in taste and flavor between these two treatments (T.L.G., unpublished data), even though they did not differ in the concentrations of 3-alkyl-2-methoxypyrazines.

Because our results from the Frontenac wine artificially infested with *H. axyridis* and Leon Millot did not support the hypothesis that 3-alkyl-2-methoxypyrazines are the main factors of *H. axyridis*-related taint in wine, we sampled four commercial Frontenac wine samples from grapes with natural levels of *H. axyridis* infestations from the same vineyard in Hastings, MN (**Table 6**). In 2001 and 2002, the presence of the off-flavor in

Table 3. Recovery Tests ($n = 3$) for *Harmonia axyridis*-free Frontenac Wine Spiked with 3-Alkyl-2-methoxypyrazines

analyte added (ng/L)	initial analyte (ng/L)	measurement after spike (ng/L)	95% CL (ng/L)	recovery of spiked sample (%)
		IPMP		
10	0	11.54	2.87–20.21	115.42
25	0	20.57	12.27–28.88	82.30
50	0	54.93	45.95–63.90	109.85
100	0	93.97	81.25–106.68	93.97
		SBMP		
10	1.04	13.54	9.57–17.52	125.04
25	1.04	23.48	19.67–27.28	89.75
50	1.04	52.05	48.06–56.05	102.02
100	1.04	99.24	93.27–105.21	98.19
		IBMP		
10	2.01	10.55	5.83–15.27	85.40
25	2.01	20.89	16.40–25.38	75.53
50	2.01	47.43	42.81–52.04	90.83
100	2.01	99.58	92.43–106.73	97.58

Table 4. Concentration of 3-Alkyl-2-methoxypyrazines ($n = 4$) in Frontenac Wine from Grapes with Increasing Levels of *Harmonia axyridis* Infestation

treatment[a]	mean IPMP[b] (ng/L) (SE)[c]	mean SBMP (ng/L) (SE)	mean IBMP (ng/L) (SE)
0	2.63 (1.33)a	1.04 (1.04)a	1.92 (1.48)a
1	2.24 (1.22)a	7.05 (0.43)b	7.51 (0.95)b
3	bql[d]	bql	0.65 (0.45)a
8	3.36 (0.59)a	3.13 (1.58)a	bql

[a] Treatments are the number of *H. axyridis* adults per kilogram of grapes. EEP (100 ng/L) was used as internal standard. [b] Mean concentrations in the same column followed by the same letters do not differ significantly ($p = 0.05$); protected least significant difference test (LSD). [c] SE = standard error. [d] bql = below quantification limits.

Table 5. Concentration of 3-Alkyl-2-methoxypyrazines ($n = 3$) in Leon Millot Wine from Grapes with Low and High Levels of *Harmonia axyridis* Infestation

Leon Millot[a]	mean IPMP[b] (ng/L) (SE)[c]	mean SBMP (ng/L)	mean IBMP (ng/L)
20%	6.10 (0.01)a	bql[d]	bql
50%	7.17 (1.60)a	bql	bql

[a] Leon Millot (2004, Hastings, MN) from grapes protected by a floating row cover (20% of clusters infested with at least one *H. axyridis* adult) and from grapes not protected (50% of clusters infested with at least one *H. axyridis* adult). EEP (100 ng/L) was used as internal standard. [b] Mean concentrations in the same column followed by the same letters do not differ significantly ($p = 0.05$); Student's *t* test (*t* test). [c] SE = standard error. [d] bql = below quantification limits.

Table 6. Concentration of 3-Alkyl-2-methoxypyrazines ($n = 4$) in Frontenac Wine from Grapes with Natural Levels of *Harmonia axyridis* Infestation

Frontenac[a]	mean IPMP[b] (ng/L) (SE)[c]	mean SBMP (ng/L) (SE)	mean IBMP (ng/L) (SE)
2001	1.45 (1.45)a	bql[d]	bql
2002	1.39 (1.39)a	bql	3.88 (0.76)a
2003	5.98 (2.00)a	bql	8.73 (4.36)ab
2005	4.64 (0.59)a	bql	13.83 (1.05)b

[a] Frontenac wine samples of four years from Hastings, MN. EEP (100 ng/L) was used as internal standard. [b] Mean concentrations in the same column followed by the same letters do not differ significantly ($p = 0.05$); protected least significant difference test (LSD). [c] SE = standard error. [d] bql = below quantification limits.

wine was first detected in Minnesota, but its origin was unknown by the grape grower. Since 2003, when research in Ontario, Canada (*2*), and Minnesota (*1*) started to associate the off-flavor in wine with the presence of *H. axyridis* in vineyards, grape growers have started to monitor and control lady beetle populations. However, results in the quantification of 3-alkyl-2-methoxypyrazines showed that Frontenac from 2003 and 2005 had numerically higher concentrations of IPMP than Frontenac from 2001 and 2002, even though the mean concentrations were not statistically significant ($p = 0.12$) (**Table 6**). SBMP concentrations were below quantification limits. IBMP concentrations in Frontenac 2005 were higher than in 2001 and 2002, but similar to the IBMP concentrations in 2003 ($p = 0.02$) (**Table 6**). Pickering et al. (*8*) reported a 50% reduction of IPMP and IBMP concentrations in wine stored for 11 months. The breakdown of these compounds may explain why we found them at lower concentration in the 2001 and 2002 Frontenac wines.

The association of 3-alkyl-2-methoxypyrazines produced in *H. axyridis* to the off-flavor in the wine produced from grapes infested with *H. axyridis* is logical because these compounds have been linked to the off-flavor that naturally appears in Cabernet Sauvignon, Cabernet Franc, Sauvignon Blanc, and Merlot (*14, 15*). Enology and viticulture researchers have investigated the effects of these compounds in wine and tried several remediation procedures for the past three decades (*14, 28*). The most studied 3-alkyl-2-methoxypyrazine in wine research is IBMP, which accounts for 90% of total concentrations of these compounds in wine depending on the grape variety (*29*). These compounds have also been identified and quantified in different species of lady beetles (*4, 7, 9*). In *H. axyridis*, the concentration of IPMP ranges from 70 (*9*) to 80% (*7*) of the total concentration of 3-alkyl-2-methoxypyrazines. Therefore, IBMP is the main compound present in wine made from grapes with natural levels of pyrazines, and IPMP is the predominant 3-alkyl-2-methoxypyrazine in lady beetles.

Table 7. Concentration of 3-Alkyl-2-methoxypyrazines ($n = 3$) in Frontenac and Model Wine Containing Adults of *Harmonia axyridis*

sample[a]	mean IPMP[b] (ng/L) (SE)[c]	mean SBMP (ng/L) (SE)	mean IBMP (ng/L) (SE)
Frontenac	132.54 (7.99)a	57.06 (2.70)a	2.47 (1.27)
Model wine	176.89 (2.07)b	136.88 (1.13)b	bql[d]

[a] Two live adults of *H. axyridis* were added to the 50 mL solution containing either Frontenac wine (*H. axyridis*-free) or model wine (12% of ethanol). EEP (100 ng/L) was used as internal standard. [b] Mean concentrations in the same column followed by the same letters do not differ significantly ($p = 0.05$); Student's *t* test (*t* test). [c] SE = standard error. [d] bql = below quantification limits.

In our study, we also found IBMP in higher concentrations than IPMP in Frontenac (**Tables 4** and **6**), but not in Leon Millot (**Table 5**). To investigate if IPMP is the major 3-alkyl-2-methoxypyrazine in *H. axyridis*, we collected headspace volatiles in Frontenac and model wine containing *H. axyridis* adults (**Table 7**). The sample preparations for SPME extraction and GC analyses for these studies followed the same methods employed for the determination of IPMP, SBMP, and IBMP in model wine and wine solutions. When we added live adults of *H. axyridis* to Frontenac and model wine, IPMP and SBMP concentrations were higher (at least 20 times) than that of IBMP (**Tables 7**). In addition, IPMP and SBMP concentrations were higher in model wine than in Frontenac at a similar ethanol concentration ($p < 0.05$) (**Table 7**). The chemical and/or physical composition of Frontenac wine may be affecting the volatilization of these compounds (*25*). These results corroborate the two previous studies (*7, 9*) that showed IPMP as the major 3-alkyl-2-methoxypyrazine in *H. axyridis*. Our results also suggest that the IBMP present in Frontenac wine may occur naturally in the grapes of this variety because it is less likely to originate from *H. axyridis* infestations.

To date, only Pickering and co-workers have identified IPMP as a key component of *H. axyridis* off-flavor in wine (*8*). In their study, adults of *H. axyridis* were added to grape juice concentrate. Their results showed that wine from grape juice infested with 10 lady beetles/L had higher concentrations of IPMP than the untreated wine. No differences in IBMP concentration were found among treatments in the same study. In our study, IPMP concentrations were not significantly different among the levels of *H. axyridis* artificial infestations in Frontenac or the levels of *H. axyridis* natural infestation in Leon Millot. In commercial 2001, 2002, 2003, and 2005 Frontenac wines, IPMP was not detected in the first two years, but that may be a result of breakdown of this compound. SBMP was present in the artificially infested wine, but it was not detected in naturally infested Frontenac and Leon Millot wines. The role of SBMP in the *H. axyridis*-related taint in wine is unknown. IBMP concentrations were not significantly different among the levels of *H. axyridis* artificial infestations in Frontenac. In commercial 2001, 2002, 2003, and 2005 Frontenac wines, IBMP was detected in lower concentrations in the first two years, but this may also be a result of compound breakdown. IBMP was not detected in Leon Millot wine under natural infestations of *H. axyridis*. In general, IBMP concentrations were below the sensory threshold (15 ng/L) for other red wines (*15*). These results do not relate IPMP, SBMP, or IBMP concentrations with the *H. axyridis*-related off-flavor in artificially infested Frontenac wine or the different levels of natural infestation in Leon Millot.

In addition, the method for extraction of 3-alkyl-2-methoxypyrazines from wine showed high linearity ($r^2 > 0.98$), low detection limit (>0.25 ng/L), good precision (RSD < 20%), and accuracy (recovery = 75–125%), which were equivalent to those of previous methods using headspace-SPME (*11, 22, 25*). Even with these desirable method parameters, we do not dismiss the possibility that IPMP, SBMP, IBMP, or their combination could contribute to the *H. axyridis*-related taint in wine. Differences in the vinification and infestation procedures, tasting panels, or extraction methods between Pickering and co-workers (*8*) and our study may have contributed to the disparity in the results between studies.

Future research should, first, focus on the determination of the primary components that could be responsible for the *H. axyridis*-related taint in wine, and, second, on the quantification or possible remediation of 3-alkyl-2-methoxypyrazines. Numerous additional compounds, such as alkaloids or terpenes, are also present in lady beetles (*5, 9, 31*) and may also contribute to the off-flavor in wine. For example, Cai et al. (*9*) identified 2,5-dimethyl-3-methoxypyrazine from live *H. axyridis* as a potential lady beetle odorant. Actually, a previous study showed that the reduction of IPMP concentration using charcoal or deodorized oak was not followed by a decrease in the off-flavor caused by the lady beetle (*30*), suggesting that other compounds may be causing the taint.

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