



Morbidity of *Harmonia axyridis* mediates ladybug taint in red wine

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Abstract

Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae; the Multicolored Asian Lady Beetle; MALB) is recognised as a novel vineyard pest in many grape and wine regions of the world due to tainting of juice and wine from excretion or extraction of 2-isopropyl-3-methoxy-pyrazine (IPMP) when beetles are harvested with the grapes during processing. This complex of off-odors and flavors in juice and wine is known as 'ladybug taint' (LBT). The use of insecticidal sprays in the vineyard has been advocated to mitigate the problem, however, the resulting dead beetles are often inadvertently incorporated in with the grape bunches during harvesting operations. The main objective of this study was to quantify the impact of dead MALB on LBT in red wine. Duplicate wines were produced from Cabernet Sauvignon with the addition of 10 beetles/L juice, added either live or at 1, 3, 7, or 60-days post-mortem. A control wine with no added beetles was also included. Finished wines were evaluated using a trained sensory panel and descriptive analysis techniques. The intensities of aroma and flavour attributes associated with LBT were highest in live beetle wines. Sensory data were inconclusive for the remaining wines, although they suggest that MALB did not affect wine quality after 3 days post-mortem and beyond. Concentrations of IPMP were strongly and positively correlated with 6 atypical aroma and flavour attributes ($r=0.902 - 0.966$), confirming the association between IPMP and LBT, and the intensity of LBT was linearly related to IPMP concentration ($LBT = 3.059 + 0.048*IPMP$; $R^2 = 0.934$). These results quantify the impact of dead MALB on wine quality and should help in establishing grape quality parameters and inform decisions regarding the use of insecticides in the vineyard.

Key words: *Harmonia axyridis*, MALB, vineyard pest, IPMP, LBT, red wine, aroma and flavour.

Introduction

Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae; the Multicolored Asian Lady Beetle; MALB) is distributed widely in the USA and Canada¹⁻³ and have also been identified in France, South America, England, Switzerland and other winemaking countries⁴. MALB negatively influence wine quality when present with the fermenting juice, contributing bell pepper, peanut, earthy/herbaceous and asparagus aromas and flavours⁵. This complex of undesirable characteristics is called 'ladybug taint' (LBT), and is resistant to common wine fining agents⁶ and stable after bottle ageing⁷.

The primary compound responsible for this taint is 2-isopropyl-3-methoxy-pyrazine (IPMP)^{7, 8}, which is a component of Coccinellidae haemolymph and is thought to serve as an alerting signal and an aggregation pheromone⁹. This compound has a very low human olfactory threshold in wine, ranging from 0.32 to 2.29 ng l⁻¹¹⁰. LBT can develop if IPMP is transferred from MALB into juice or fermenting must, however, transfer from MALB onto grapes prior to or during harvest at concentrations that elicit LBT does not appear likely¹¹. Tolerance limits for MALB at harvest, below which development of LBT is not expected, are estimated as 1250 - 1550 beetles/ton grapes, although a more conservative limit of 200-400 beetles/ton grapes has also been suggested¹¹.

Given the considerable economic significance of LBT for the juice and wine industry⁸, further examination of its origins and possible mitigation has been advocated. The use of insecticidal sprays on MALB in the vineyard has recently become widespread; however, a practical concern is that dead beetles are often incorporated in with the harvested grapes, as many remain resident within berry clusters post-mortem. This makes it challenging to assess the actual efficacy of sprays and other treatments in preventing LBT in the final wine. Recently, Pickering *et al.*¹² quantified the effect of dead MALB on IPMP concentrations in Cabernet Sauvignon wine. Wines were produced after the addition of live MALB to juice, and at 1, 3, 7 or 60-days post-mortem. IPMP concentration was substantially higher in live-beetle wines and decreased to base-line levels at approximately 6.5 days post-mortem. However, the sensory profiles of these wines were not reported, particularly with regard to LBT. The need for corroborating sensory data for the purported impact of dead MALB on wine quality is further established by the possibility that methoxy-pyrazines other than IPMP may contribute to LBT¹³. The main objectives of this study, therefore, were to determine the influence of dead MALB on LBT in red wine, and further examine the relationship between IPMP and LBT.

Materials and Methods

Preparation of samples: The wines described in Pickering *et al.*¹² were used for all sensory and chemical analysis. Commercial Cabernet Sauvignon juice concentrate (Californian Connoisseur, Vineco International Products Ltd., St. Catharines, ON) was used to make the base juice. After rehydration following manufacturer's instructions, the juice was separated into 10-litre glass carboys and inoculated with yeast strain EC1118 (Lallemand Inc., Santa Rosa, California) at 300 mg l⁻¹. MALB were added to each carboy at a rate of 10 beetles/litre either as live beetles or 1, 3, 7 or 60 days post-mortem. A control condition followed the same protocol without beetles. Live MALB were obtained from the biological control laboratory at University of Guelph as unmated adults. They were euthanized with CO₂ gas and then transferred into a plastic container with the top closed with nylon to allow for air circulation to partially reflect environmental conditions within berry clusters in a vineyard. The container was stored at room temperature (approx. 21°C) until the beetles were added to the fermentation vessels. All treatments were duplicated and fermentations were carried out at 18°C. Wines were monitored daily for temperature and °Brix and, at the completion of primary fermentation, were racked, sulfited, cold-stabilised and stored at -2°C until required for analysis.

Chemical analysis: The basic physicochemical composition of the wines was determined after Iland *et al.*¹⁴. IPMP was determined using solid-phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME-GC-MS), and quantitation was achieved using an internal deuterated (OD₃) IPMP standard ([²H₃] IPMP). Full details on this method are given in Kotseridis *et al.*¹⁵. Results are shown in Table 1.

Sensory analysis

Descriptor generation and panel training: The panel consisted of 6 female Brock University wine students and staff ranging in age from 25 to 54 yrs. All panellists had previously participated on multiple wine descriptive analysis panels, including panels evaluating LBT. One training session (2 hrs) was held. Minimal information about the nature of the study was given to the participants in order to reduce potential bias. During the first half of the training session the panel was presented with samples from four of the six wine treatments. They were also given a list of descriptors previously used to profile LBT in Cabernet Sauvignon from an earlier study¹⁶. The panel was instructed to generate aroma and flavour descriptors for each wine, using the list as a guide as needed. Led by the panel leader, the list of descriptors was discussed and the group removed any redundant or overlapping terms. Terms that were used by only one member were removed, by panel consensus, and the final lexicon was developed. In the second half of the training session, candidate reference standards based on Spink¹⁶ were presented to the panel and refined to meet the group's criteria as suitable standards for the descriptors generated in the first half of the session.

A 15 cm line scale was also developed for each descriptor, with endpoints indented 1 cm from the end of the scale to avoid endpoint effects¹⁷. The endpoints of the scale were labelled "absent" on the left and "very high" at the right hand end mark. During the training session the panel gained experience evaluating the standards and the method of data collection. The final lexicon and list of reference standards is given in Table 2.

Table 1. Physicochemical composition of Cabernet Sauvignon wine¹.

	Stage of <i>Harmonia axyridis</i> addition to wine ²					
	Control (no beetles)	Live beetles	Dead 1 day	Dead 3 days	Dead 7 days	Dead 60 days
pH ³	3.44±0.04	3.54±0.06	3.47±0.08	3.49±0.02	3.51±0.07	3.57±0.04
Titrateable acidity (g.l ⁻¹) ³	6.74±0.03	6.24±0.03	6.71±0.04	6.74±0.06	6.64±0.05	6.21±0.04
Ethanol (% v/v) ³	11.5±0.2	10.9±0.5	11.9±0.4	11.4±0.2	11.2±0.3	10.8±0.4
IPMP ⁴ (ng.l ⁻¹) ⁵	14.5±0.4	59.4±4.7	21.6±0.5	20.3±0.7	14.6±0.7	14.7±0.05

¹Adapted from Pickering *et al.*¹²; ²beetles added at 10 beetles/litre juice; ³data represent the mean values of triplicate measurements of duplicate fermentation replicates ± standard deviation; ⁴2-Isopropyl-3-methoxy-pyrazine; ⁵data represent the mean values of duplicate measurements of duplicate fermentation replicates ± standard deviation.

Table 2. Cabernet Sauvignon wine aroma and flavour descriptors with corresponding reference standards.

Descriptor	Reference standard ¹
Red berry	<ul style="list-style-type: none"> • 5 ml fresh strawberry purée • 15 ml fresh raspberry purée • 15 ml fresh cherry purée
Vanilla/Caramel	<ul style="list-style-type: none"> • 50 ml caramel syrup (Smucker'sTM Zehrs St. Catharines, ON) • 3 drops vanilla extract (Club HouseTM Sobeys St. Catharines ON)
Canned green vegetables	<ul style="list-style-type: none"> • 10 ml canned asparagus juice² • 10 ml canned green bean juice² • 10 ml canned pea juice²
Green pepper	<ul style="list-style-type: none"> • 10 grams crushed fresh green pepper
Candy	<ul style="list-style-type: none"> • 10 ml cream soda (Cadbury Beverages Canada Inc., Mississauga, ON) • 1 TwizzlerTM red licorice (Hershey Canada Inc. Mississauga, ON)
Earthy/Peanut/Musty	<ul style="list-style-type: none"> • 1 tablespoon peanut butter²
Jammy	<ul style="list-style-type: none"> • 3 tablespoons wildberry jam (Smucker'sTM, Sobeys, St. Catharines, ON)

¹All standards made in 50ml base Cabernet Sauvignon wine (Californian Connoisseur®, Vineco International Ltd., St. Catharines, ON) unless otherwise stated; ²EqualityTM brand, Food Basics, St. Catharines, ON.

Data collection and statistical analysis: The wines were assessed in three sessions in the sensory laboratory at the Cool Climate Oenology and Viticulture Institute at Brock University. The six wines were evaluated in triplicate for aroma and flavour intensity under red lights in individual white tasting booths. The wines were presented at room temperature in covered ISO tasting glasses coded with random 3-digit numbers. The order of presentation was randomized within each flight. The data were collected using the Compusense™ *five* software (Compusense Inc., Guelph, Ontario, Canada). Before each evaluation session, the judges were asked to re-familiarize themselves with the reference standards, which were available during data collection if needed. The aroma and flavour descriptors were evaluated for each wine with a two-min minimum break between samples and a minimum one hour break between flights. Panellists were also given the option of noting additional descriptors that did not appear on the ballot.

Data were analyzed using the ANOVA procedure within XLSTAT® version 7.5.2 (Addinsoft, 40, rue Damrémont, 75018 Paris, France). For sensory data, judge, treatment and their interaction were included as the independent variables. If $p(F)$ was <0.05 , Tukey's HSD was used as the means separation test. Principal Component Analysis (PCA) without rotation was also performed on the sensory data.

Results and Discussion

Sensory data: The control wine was deemed severely oxidised by the panel (frequency of use of "oxidised" and related descriptors = 12) and was not included in further analysis. The origin of the oxidation was technical error in SO_2 monitoring post-fermentation. Table 3 gives the mean intensity scores for the aroma and flavour attributes assessed, and Fig. 1 shows the factor loadings and scores from PCA of the sensory data.

Factors 1 and 2 account for 73% of the variation in the data. Factor 1 is positively loaded with positively correlated aroma and flavour attributes previously associated with MALB^{5,7} and negatively loaded with both grape and winemaking-derived aroma and flavour attributes. Thus, it may be conceived of as a LBT dimension. Factor 2 is not well defined by any specific eigenvector(s). Live beetle wines (indicated by oval) are grouped together and separated from others based on their position along

Factor 1, reflecting their higher intensity scores for MALB-associated characters (Table 3).

Wines from post-mortem additions show no clear groupings either by treatment or replicate and are dispersed throughout the sensory space. The 1-day post-mortem wines tend towards slightly higher intensity score for LBT-associated attributes (Table 3 and Fig. 2) and lower intensity for vanilla/caramel (Table 3), suggesting perceptible influence from MALB, although these differences are not statistically significant. Conversely, the 3-day post-mortem wines tend towards lower scores for LBT characteristics and higher scores for both grape- and winemaking-derived attributes, suggesting that influence from MALB addition is not perceptible after that time. Wines fermented in the presence of live MALB, also at 10 beetles/litre, have previously been characterised by similar sensory profiles as described here⁵. Additionally, live beetle wines in the current study were scored lower for fresh berry aroma, fresh berry flavour and vanilla/caramel attributes, suggesting a masking effect by LBT on varietal- and winemaking-derived characteristics, in agreement with Pickering *et al.*^{5,7}.

IPMP:sensory relationships: As shown in Table 1 and discussed in Pickering *et al.*¹², fermentation in the presence of live MALB leads to substantially higher IPMP concentration in the finished wines. When beetles are added at 60 and 7 days post-mortem, there is no increase in IPMP. Pearson's correlation coefficient was calculated for each sensory attribute score and IPMP concentration for all treatment wines. IPMP was strongly and positively associated with earthy/musty/peanut aroma ($r = 0.958$), green pepper aroma ($r = 0.966$), canned green vegetable aroma ($r = 0.942$), earthy/musty/peanut flavour ($r = 0.946$), canned green vegetable flavour ($r = 0.937$) and green pepper flavour ($r = 0.902$). Intensity scores for these 6 attributes were averaged for each wine to construct a single value for LBT (Fig. 2). This was then modeled using linear regression to yield the equation $LBT = 3.059 + 0.048 * IPMP$, with a corresponding R^2 of 0.934.

While caution should be applied in interpreting small data sets such as those presented here, overall these results further support the hypothesis that IPMP is the causal compound of LBT and provide some guidance for estimating intensity of LBT from IPMP concentration in affected wines. Interestingly, wines at 7 days

Table 3. Aroma and flavour intensity scores, F-ratios and groupings for Cabernet Sauvignon wine produced with addition of 10 *Harmonia axyridis* beetles/litre juice.

Attribute	F-ratio & Sig.	Treatment				
		Live beetles	Dead 1 day	Dead 3 days	Dead 7 days	Dead 60 days
Fresh red berry aroma	1.54	2.41a	3.61a	3.99a	3.58a	3.47a
Candy aroma	0.65	1.85a	2.60a	2.84a	3.01a	2.52a
Jammy red berry aroma	3.67**	1.29b	2.52ab	2.98a	2.21ab	2.26ab
Vanilla/Caramel aroma	2.43	0.64a	0.701a	1.68a	1.54a	1.66a
Earthy/Musty/Peanut aroma	4.42**	7.66a	5.11ab	4.14b	4.77b	4.56b
Green pepper aroma	1.75	4.99a	3.54a	3.46a	3.43a	3.70a
Canned green vegetable aroma	1.77	5.40a	3.77a	3.63a	3.92a	3.96a
Fresh red berry flavor	5.83***	1.82b	3.94a	3.96a	4.17a	4.04a
Jammy red berry flavor	3.11*	1.89b	2.57ab	3.07ab	3.47a	2.16ab
Candy flavor	3.87**	0.81b	0.97b	2.08a	1.48ab	1.14ab
Earthy/Musty/Peanut flavor	4.92**	6.57a	4.31b	3.43b	4.14b	3.87b
Canned green vegetable flavor	6.68***	6.21a	4.32b	3.49b	4.19b	3.79b
Green pepper flavor	2.37	5.01a	3.92ab	3.56ab	2.99b	3.82ab

* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

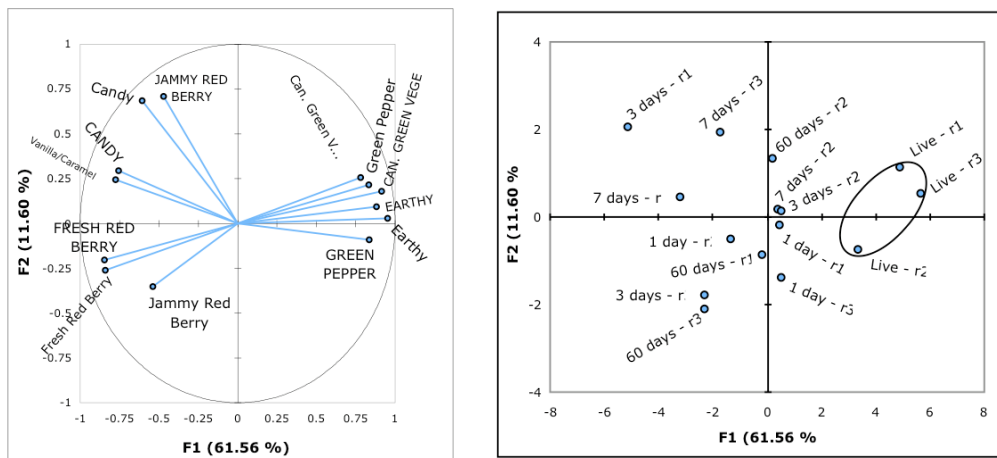


Figure 1. Factor loadings and scores from Principal Component Analysis of aroma and flavour intensity ratings elicited by Cabernet Sauvignon wine produced with addition of 10 *Harmonia axyridis* beetles/litre juice (right figure: each data point represents the factor score from the ratings of 6 judges for each of the triplicate assessments (r1-r3). Labels indicate whether beetles were added live or at specific time post-mortem).

post-mortem and the control wines contained 14.5 ng l⁻¹ IPMP, above the human detection threshold in red wine of 1.0 and 2.3 ng l⁻¹ for orthonasal and retronasal evaluation respectively¹⁰. Establishment of sensory difference thresholds for IPMP would be valuable in more fully understanding and predicting the influence of MALB in wines with significant grape-derived methoxypyrazine content.

Further Considerations and Conclusions

These sensory data, in combination with the analytical results reported in Pickering *et al.*¹² allow for more robust conclusions concerning the influence of MALB morbidity on wine quality. Both studies indicate that live MALB have a greater impact than dead beetles, presumably through active excretion of IPMP-laden haemolymph into the juice/wine matrix. When dead beetles are added to juice there is no clear evidence of LBT developing in the wine if they have been dead for 3 days or longer, although the absence of usable sensory data for the control wines in this study

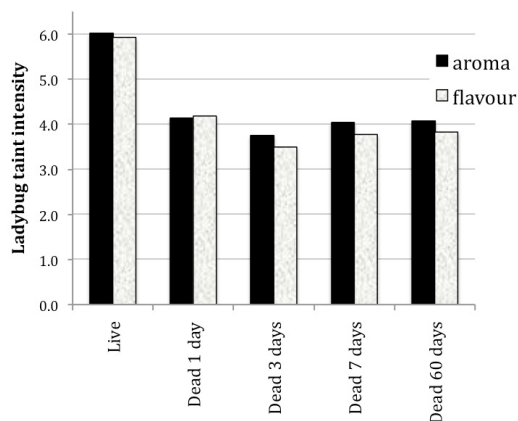


Figure 2. Intensity of ladybug taint elicited by Cabernet Sauvignon wine produced with the addition of 10 *Harmonia axyridis* beetles/litre juice added live or at specific periods post-mortem (ladybug taint is the average aroma or flavour scores for green pepper, earthy/musty/peanut and canned green vegetable attributes as assessed by 6 judges over triplicate evaluations).

complicates this interpretation. As IPMP levels return to baseline at approximately 6.5 days post-mortem¹², we recommend that industry adopt this more conservative period as its guideline for when dead MALB incorporated with grapes at harvest may no longer be a risk factor for LBT in subsequent wines.

We speculate that MALB will have less of an impact post-mortem in white wines, as these grapes are typically pressed soon after they are received and beetles removed with the marc. Conversely, red winemaking techniques that employ extended skin maceration or thermovinification have the potential to yield greater extraction of IPMP from MALB still present. Such practises are discouraged when fruit is known to contain MALB.

Spraying with insecticide is increasingly used for controlling MALB and, given the different responses of live and dead MALB observed here, can be a valid intervention. In Canada, there are currently only 2 registered sprays for MALB - Ripcord 400EC (Cypermethrin) and Malathion 500E - with 7 and 3-day pre-harvest intervals (PHI), respectively¹⁸. A limitation of these sprays is that long PHIs mean greater opportunity for MALB to reinfest a vineyard, and they may still be capable of negatively affecting wine quality post-mortem if sprayed 3 days or earlier prior to harvest. Given this ability for MALB to contribute to LBT post-mortem, we encourage (i) removal of dead beetles from the vine prior to harvest where possible, and (ii) more research on and consideration of repellency sprays and related strategies.

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