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Determination of the critical stages of processing and tolerance limits for *Harmonia axyridis* for 'ladybug taint' in wine

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Summary

'Ladybug taint' (LBT) has recently been reported in some wines from North America, and is associated with 2-isopropyl-3-methoxypyrazine (IPMP), produced by Harmonia axyridis Pallas (the Multicolored Asian Lady Beetle - MALB) when they are incorporated into the winemaking process. It is not known when IPMP is transferred from MALB (e.g. in the vineyard onto grapes or during must processing) nor what minimum MALB densities are required for production of LBT in the final wines. This study sought to clarify these issues through a series of three trials. In the first, MALB were added to 'Riesling' grapes or must at different stages of processing (harvest, crush/destem, pressing or directly to juice), and the resultant wines were analysed chemically and by paired-comparison sensory difference tests.

The presence of MALB during processing had minimal effect on the basic composition and spectral properties of the wine. Concentrations of IPMP were < 5 ng·l^{-1} for all wines except those produced after the direct addition of MALB to the juice (10.3 ng·l^{-1}). Sensorially, control wines (no added MALB) could be differentiated from wines made after MALB were added at crushing/destemming (at 3 beetles per kg grapes), whole bunch pressing and when added directly into the juice, but not when MALB were added and subsequently removed from a simulated harvest treatment or when added during crushing/destemming at 0.3 beetles per kg grapes. In trials 2 and 3, sensory detection thresholds for LBT were determined for white and red wines produced with known densities of MALB. Estimates of 'tolerance limits' in the vineyard were then calculated using regression models, and correspond to 1530 and 1260 beetles per t grapes for white and red wines respectively. However, given the range of grape and wine processing options available to producers, many of which are not accounted for in this study, we recommend that a more conservative limit of 200-400 beetles per t grapes may be appropriate. These results should assist in directing appropriate interventions in the vineyard/winery, and provide baseline targets for reducing MALB density to avoid development of LBT.

K e y w o r d s : Multicolored Asian Lady Beetle; vineyard pest; threshold; wine quality; fault.

Introduction

Harmonia axyridis (Pallas) *(Coleoptera: Coccinellidae)* (the Multicolored Asian Lady Beetle) (MALB) is found throughout North America, including Texas, Missouri, Ohio, Oregon, Washington, British Columbia, Quebec and Ontario (Day *et al.* 1994, Hoebeke and Wheeler 1996, Nalepa *et al.* 1996), and has also been introduced into France and other winemaking countries (Pickering and Lin 2006). There have been anecdotal reports from wineries in some parts of North America of an unusual aroma and flavour, suggestive of crushed lady beetles, in some wines from the area, often concurrent with the observation of high numbers of MALB in vineyards and on the fruit at harvest (Pickering *et al.* 2004). MALB have been shown to adversely affect wine quality when present with the fermenting juice. Specifically, they can contribute peanut, bell pepper and asparagus aromas and flavours in white wine, and peanut, asparagus/bell pepper, and earthy/herbaceous aromas and flavours in red wine (Pickering *et al.* 2004). In addition, a general loss of varietal aroma and flavour was shown, and the sensory impact on final wine quality increased with increasing numbers of added MALB.

This 'ladybug taint' (LBT) is stable after bottle ageing (Pickering *et al.* 2005) and generally resistant to common wine fining agents (Pickering *et al.* 2006 a) although basic wine composition is not affected by MALB (Pickering *et al.* 2005). The data of Pickering *et al.* (2005) strongly implicates 2-isopropyl-3-methoxypyrazine (IPMP) as the causal compound of this taint, which is further collaborated by the findings of Pickering *et al.* (2006 b). IPMP is a component of the haemolymph of *Coccinella* (Al Abassi *et al.* 1998), has been identified in MALB (Cudjoe *et al.* 2005) and has a very low human olfactory threshold, in the order of 2 ng·l^{-1} (Buttery *et al.* 1969; Seifert *et al.* 1970). Coccinellids possess a reflex bleeding response of haemolymph when stressed (Abassi *et al.* 1998, Laurent *et al.* 2001, Koch 2003), and it has been speculated that MALB influence wine quality via transfer of haemolymph onto grapes, or directly into juice or must if the beetles become incorporated

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with the harvested fruit (Pickering *et al.* 2004, 2005). LBT has resulted in significant economic losses for vineyards and wineries (Pickering *et al.* 2006 b), and further investigations aimed at improving understanding of the origins of this off-flavour and development of preventative and remedial treatments have been urged. It is unclear *when* during the harvesting or juice/wine processing operations that MALB activity results in taint of the finished wine. For instance, it is not known to what extent IPMP release from MALB is an active process (e.g. a reflex bleeding response of haemolymph onto grapes) or a passive one (e.g. through rupture of the carapace during crushing/pressing or ethanol extraction during fermentation). Finally, the minimum number of beetles required to produce a perceptible taint in wine has not been established. Together, this information should assist in directing appropriate interventions in the vineyard/winery, including the setting of 'rejection' limits for MALB in harvested fruit, and provide baseline targets against which the efficacy of treatments aimed at reducing MALB densities could be evaluated.

Thus, the main objectives of this study, conducted in three trials, were (i) to determine at what stage(s) during grape and wine processing the taint is introduced, and (ii) to provide an estimate on how many beetles are required to produce a discernable taint in finished wine.

Material and Methods

Preparation of samples

Trial 1: Critical stages of processing: 690 kg of 'Riesling' were sourced from a commercial vineyard in Niagara, Canada, believed to be free from MALB and with a history of low- no occurrences of MALB. The fruit was hand picked and placed in 23 l plastic containers and transported to the Brock University winery where all bunches were carefully hand-sorted and any beetles discovered (n = 2) were carefully removed and disposed of. The following treatments were then instigated using the winery's microvinification equipment:

Treatment 1: Control: A portion of the fruit was processed without any addition of MALB using standard microvinification techniques, including crushing, destemming and pressing in a bladder press (to 2 bar). Fermentations were conducted in triplicate in closed 20 l carboys.

Treatment 2: Agitation: The potential for MALB to influence final wine quality through secretion onto grapes before crushing/destemming was examined. MALB were sourced from KCMS Inc (Grimsby, ON L3M 2P2, Canada) and species identity confirmed using the criteria given in Pickering *et al.* (2004). Live MALB were added at 3 beetles·kg^{-1} grapes to 23 l plastic pails containing 10 kg of intact fruit. The closed pails were inverted and rolled vigorously for 45 sec using a standardised protocol to approximate the duration and quality of disturbance that might be expected during mechanical harvesting of grapes. Pails were then opened and all beetles carefully removed by hand. The fruit was then processed and vinified per Treatment 1.

Treatment 3: Crush/destem: The ability of MALB to influence final wine quality after incorporation into the crushing/destemming process was examined. Live beetles were processed as described in Treatment 2, except: (i) a second level of beetle addition was included (0.3 beetles·kg^{-1} grapes) and (ii) beetles were not removed after agitation in the pails.

Treatment 4: Whole bunch press: The possibility that LBT may be avoided or moderated by skipping the crush/destem step was examined. Fruit was processed per Treatment 1 without crushing/destemming. Live MALB were then added with the grapes to the press at a rate of 3 beetles·kg^{-1}, and incorporated evenly into the volume.

Treatment 5: Direct addition to juice: Red wine production was partially simulated by the incorporation of beetles to the pressed juice after it had been cold-settled. They were removed after fermentation at the first racking. The rate of addition was calculated based on the juice yield obtained after pressing so as to be equivalent to 3 beetles/kg grapes, and thus allow comparison with results from the former treatments.

Wines from all treatments were fermented to dryness. MALB activity was monitored closely during and after their addition to the grapes/juice, and no loss of any beetles from flight was observed.

Trial 2: Tolerance level - white wine: The crush/destem (3 beetles·kg^{-1}) wine from Trial 1 (LBT) was blended in different proportions with the control wine (C) to create a concentration series of 'ladybug taint' for the determination of ortho-nasal thresholds. Based on bench-testing, five blends were prepared: (i) LBT 20 %, C 80 %, (ii) LBT 35 %, C 65 %, (iii) LBT 50 %, C 50 %, (iv) LBT 70 %, C 30 % and (v) LBT 100 %.

Trial 3: Tolerance level - red wine: Wines previously described in Pickering *et al.* (2004) were prepared from Red Bergamais™ juice concentrate (Vineco International, St Catharines, Ontario) from South American grapes, and were re-hydrated according to manufacturer's directions. MALB were sourced from the local area and screened for identity as outlined above. Live MALB were added to re-hydrated juice in 20 l closed glass carboys at rates of 0 (C) and 10 (LBT) beetles per l of juice. Three 20 l replicates of the LBT wine and four 20 l replicates of the control wine were prepared and fermented to dryness using standard microvinification techniques. After fermentation they were racked (including removal of beetles), sulfited, cold stabilized, and stored in a cellar at 14 °C until required.

These two wines were blended in different proportions to create a concentration series of LBT for the determination of ortho-nasal thresholds. Based on bench-testing, five blends were prepared: (i) LBT 5 %, C 95 %, (ii) LBT 10 %, C 90 %, (iii) LBT 30 %, C 70 %, (iv) LBT 50 %, C 50 %, and (v) LBT 90 %, C 10 %.

Chemical analysis: Basic chemical and spectral analysis was conducted on the wines from trial 1 at bottling. Titratable acidity (TA), residual sugar (RS), free SO_2 (FSO2), total SO_2 (TSO2) and spectral estimates of phenolics, browning and pinking were assessed using

the methods of Iland (1988). Ethanol was determined using Gas-Chromatography and a 7-point calibration curve ($R^2 = 0.998$) after Nurgel *et al*. (2004). TA, pH and spectral measurements were repeated after two years of bottle aging. IPMP and 2-Methoxy-3-isobutylpyrazine (IBMP) were determined in duplicate for each treatment and fermentation. Wine samples were concentrated in a C-18 SPE cartridge and eluted by ethyl acetate. The ethyl acetate extract was analyzed by GC-MS using a DB5-MS column as described in Pickering *et al.* (2005). The limit of quantitation was 5 $ng \cdot l^{-1}$.

The composition of the control and LBT wines from trial 3 were also determined using these methods. Results for control and LBT wines, respectively, were: pH: 3.39 + 3.39; TA ($g \cdot l^{-1}$): 6.77 + 6.71; ethanol (% v/v): 12.65 + 12.60; RS ($g \cdot l^{-1}$): 5.37 + 4.10; IPMP ($ng \cdot l^{-1}$): < 5 + 20.9; IBMP ($ng \cdot l^{-1}$): < 5 + < 5. Data for all chemical analytes was analyzed using the ANOVA procedure within XLSTAT© version 7.5.2 (Addinsoft, 40, rue Damrémont, 75018 Paris, France) with treatment, fermentation replicate and their interaction as independent variables. If p (F) was < 0.05, Tukey's Protected HSD was used as the means separation test.

Sensory analysis: Sensory evaluation for all trials was conducted in the custom Sensory Evaluation Lab at Brock University's Cool Climate Oenology and Viticulture Institute. The lab is equipped with individual partitioned white booths, red lighting, positive pressure, and Compusense™ software (C5V4, Guelph, Ontario, Canada). ISO tasting glasses were used for all evaluations.

Trial 1: Difference tests were conducted in which wines from each of the treatments with MALB additions (agitation, crush/destem - 0.3 $beetles \cdot kg^{-1}$, crush/destem - 3 $beetles \cdot kg^{-1}$, whole bunch press, and direct addition) were compared individually with control wines (no beetles) using triangle tests. 16 panellists evaluated the various sample set combinations ortho-nasally to give a total n of between 21 and 45 for each combination. The evaluations took place over four sessions, with only one set (*i.e.* 3 glasses) presented at a time, and a minimum 10 min break enforced between each sample set. The number of correct responses was compared with the probability table (Statistical Chart 3) in Poste *et al.* (1991).

Trials 2 and 3: A detection threshold for ortho-nasal aroma was determined by 20 panellists and duplicate assessments using an ascending, forced-choice paired comparison paradigm (Lawless and Heymann 1998). Ortho-rather than retro-nasal assessment was chosen as the former mode appears more sensitive for detecting LBT (Pickering *et al.* 2004). Five pairs (sets) of samples, each consisting of one control wine and one of the LBT:C blends were presented per session. The order of presentation of each set within a session was from lowest LBT content to highest, and the presentation order within each set was balanced. Panelists were asked first to smell and familiarize themselves with two labeled reference wines; one tainted (a LBT sample) and the other a sample of control wine. They were then instructed to smell each wine within each paired set and identify the sample that had ladybug taint. A regression line was fitted for the resultant data using the 'best-fit' option within XLSTAT, and the threshold was determined to be at 75 % correct response (*i.e.* 50 % above chance) per convention (Lawless and Heymann 1998).

Results and Discussion

Trial 1: Critical stages of processing: Fermentations proceeded to 'dryness' without incident. Control wines had slightly higher ethanol and RS concentrations than wines made with MALB addition, and relatively small differences are apparent between some of the MALB treatments for these analytes (Table). TA varied across all treatments (range = 1.79 $g \cdot l^{-1}$), with the whole bunch press wine showing the highest concentration. A similar pattern of results was obtained after two years bottle aging, where concentrations ranged from 9.5 to 11.6 $g \cdot l^{-1}$ (data not shown). pH did not differ significantly across treatments at bottling or after aging (range 3.0 - 3.1, data not shown). Small differences in FSO2 and TSO2 concentration were observed between treatments, but these are unlikely to be of significance for the resultant sensory analyses. After aging, FSO2 was < 1.7 $mg \cdot l^{-1}$ for all wines and the average TSO2 concentration was 29.7 ± 5.6 $mg \cdot l^{-1}$, with minor differences between treatments noted (data not shown). Small differences in A280 nm and A320 nm values - estimates respectively of flavonoid and hydroxycinnamate content - are observed, with the whole bunch press wines showing lower values for both measures, as expected. A very similar pattern of responses was observed after bottle aging for both A280 nm (5.977 ± 0.353) and A320 nm (5.139 ± 0.419) values (data not shown). Whole bunch press wines also showed less browning (A420 nm) at bottling, although no differences between treatments were found after aging (F = 1.01, p = 0.486), and small and likely oenologically insignificant differences were found between treatments for A520 nm, a measure of 'pinking' in white wines. Overall, these data are in general agreement with Pickering *et al.* (2005), who reported no or small changes in basic wine composition and spectral measures of quality after MALB were added to and fermented with white grape juice.

IPMP and IBMP concentrations were below the limit of quantitation (5 $ng \cdot l^{-1}$) for all wines except for samples from the direct addition treatment, where IPMP concentration was 10.3 ± 0.6 $ng \cdot l^{-1}$. However, sensorially, control wines could be discriminated from crush/destem (3 $beetles \cdot kg^{-1}$) (p = 0.000), whole bunch press (p = 0.000) and direct addition (p = 0.000) wines, but not from the agitation (p = 0.079) or crush/destem (0.3 $beetles \cdot kg^{-1}$) (p = 0.399) wines. Supporting these findings, informal assessment of the wines by a small panel familiar with LBT noted the presence of the taint in the crush/destem (3 $beetles \cdot kg^{-1}$), whole bunch press and direct addition wines, and its absence in the control, agitation and crush/destem (0.3 $beetles \cdot kg^{-1}$) treatments. The methoxypyrazine data for the direct addition wines are comparable to that reported by Pickering *et al.* (2005) in white wine, who reported IPMP and IBMP concentrations of 12.3 and < 5 $ng \cdot l^{-1}$, respectively, when MALB were added at a rate of 1 beetle per litre of juice. These authors also

Table

Basic chemical composition and spectral properties of 'Riesling' wines at bottling

	Control	Agitation	Crush/destem (0.3 beetles·g^{-1})	Crush/destem (3 beetles·g^{-1})	Whole Bunch Press	Direct Addition
Ethanol (% v/v)	**13.2** *e* ± 2.0	**12.9** *c* ±0.5	**12.8** *b* ±2.2	**12.6** *a* ±0.8	**12.6** *a* ±2.0	**13.0** *d* ±1.6
Titratable acidity (g·l^{-1})	**12.2** *d* ±1.0	**12.4** *e* ±0.8	**11.9** *b* ±1.3	**12.0** *c* ±0.9	**13.0** *f* ±1.3	**11.2** *a* ±0.3
Residual sugar (g·l^{-1})	**4.0** *d* ±9.4	**2.6** *ab* ±44.7	**2.1** *a* ±32.5	**2.9** *abc* ±24.5	**3.7** *cd* ±7.6	**3.2** *bc* ±14.7
Free SO_2 (mg·l^{-1})	**14.2** *a* ±14.0	**20.5** *c* ±5.3	**17.5** *b* ±3.3	**17.5** *b* ±6.4	**16.3** *b* ±8.9	**16.3** *b* ±7.9
Total SO_2 (mg·l^{-1})	**45.1** *a* ±7.0	**49.7** *c* ±3.7	**48.0** *bc* ±3.3	**47.6** *abc* ±1.7	**47.3** *abc* ±2.8	**46.7** *ab* ±3.2
A 280 nm[1]	**6.268** *d* ±0.695	**6.032** *b* ±5.020	**6.150** *c* ±0.997	**6.237** *d* ±1.533	**5.428** *a* ±2.588	**6.495** *e* ±1.235
A 320 nm[1]	**5.825** *d* ±0.321	**5.283** *b* ±6.631	**5.718** *c* ±0.745	**5.753** *c* ±0.775	**4.702** *a* ±4.934	**5.725** *c* ±0.896
A 420 nm[2]	**0.076** *d* ±1.972	**0.076** *d* ±1.790	**0.072** *b* ±4.293	**0.072** *b* ±1.690	**0.068** *a* ±4.054	**0.075** *c* ±5.285
A 520 nm[2]	**0.021** *d* ±3.613	**0.019** *c* ±3.928	**0.018** *b* ±2.923	**0.018** *b* ±4.454	**0.021** *a* ±0.000	**0.020** *c* ±8.427

All data represent the mean values of duplicate measurements of triplicate wines ± cv (%); for each analyte means with the same letter do not differ significantly between treatments (Tukey's Protected $HSD_{0.05}$); [1] measured using a 1 mm path length and converted to 10 mm; [2] measured using a 10 mm path length.

showed that LBT could be clearly detected by a trained sensory panel in both white and red wines fermented with 1 and 10 MALB·l^{-1} of juice. It is unclear in our study what the relative importance is of the presence of ethanol (and its solvent properties) and length of time MALB are in contact with the must/wine in accounting for the higher concentration of IPMP in the direct addition wines.

These results indicate that MALB activity on grapes prior to and during harvest may have minimal or no impact on LBT in the subsequent wines. This suggests that the presence of MALB in the vineyard is not a problem *per se*, but rather they present potential for taint if incorporated with the fruit in post-harvest operations. It remains to be determined how well the simulated harvest protocol used in this trial reflects IPMP transfer during actual harvest conditions, and also any differences between hand- and machine-harvesting in this regard. However, we speculate that our protocol (closed environment, vigorous agitation) would induce greater IPMP release from MALB than typical harvest conditions.

Trials 2 and 3: Tolerance levels: Fig. 1 shows the percent correct responses from the paired comparison tests for the blended 'Riesling' wine varying in proportion of LBT and C wines. The data is fitted well (R^2 = 0.96) by the model: y = 22.0 (log x) + 89.8. The ortho-nasal sensory threshold (75 % correct responses) calculated from this regression equation corresponds to a 51 % blend of LBT:C. The number of MALB added to the grapes used in the LBT wine was 3 per kg fruit, and 51 % of 3·kg^{-1} is 1.53·kg^{-1}, which is equivalent to 1530 beetles·t^{-1} grapes. This estimate assumes that MALB behaviour, particularly in regard to transfer of IPMP, is comparable at

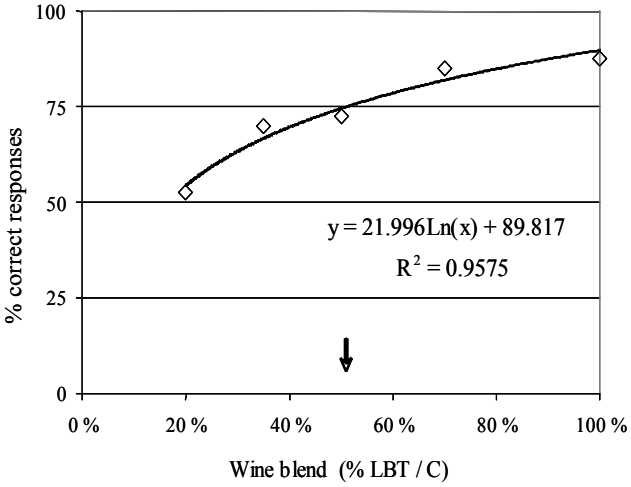

Fig. 1: % correct responses in paired comparison test for ortho-nasal evaluation of Riesling wines varying in intensity of ladybug taint (n = 40). (Composition of LBT and C wines given in text; arrow indicates calculated threshold value.)

both scales of production, and remains to be determined. 1530 beetles·t^{-1} may appear a high density of MALB, however significantly higher densities have been reported on grapes (MARTINSON 2002) and in harvest bins in North America.

Fig. 2 shows the percent correct responses from the paired comparison tests for the red wine varying in proportion of LBT and C wines. The data is fitted well (R^2 = 0.91) by the model: y = 13.8 (log x) + 35.2. The threshold (75 % correct responses) calculated from the regression equation corresponds to an 18 % blend of LBT:C. The number of

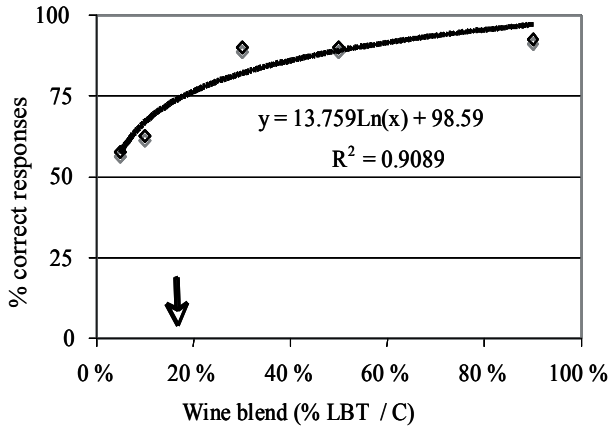

Fig. 2: % correct responses in paired comparison test for ortho-nasal evaluation of red wines varying in intensity of ladybug taint (n = 40). (Composition of LBT and C wines given in text; arrow indicates calculated threshold value.)

MALB added to the juice used in the LBT wine was $10 \cdot l^{-1}$. 18 % of $10 \cdot l^{-1}$ is $1.8 \cdot l^{-1}$. which is equivalent to 1800 beetles per 1000 L. Assuming a 70 % yield of juice, we can therefore estimate a threshold level of 1260 MALB$\cdot t^{-1}$ grapes. A number of caveats are pertinent to this estimate and are discussed below.

The estimated tolerance level for grapes intended for red wine production is less robust than for the white wine trial, as the difference between IPMP concentration elicited during post-harvest, pre-fermentation operations (particularly crushing, destemming and pressing) and that elicited during fermentation with MALB is not known. Additionally, the red wine was produced from juice concentrate, and the corresponding weight of grapes required to produce it (and thus the extrapolated threshold of MALB$\cdot t^{-1}$) can only be estimated. Caution also needs to be applied for both white and red wine in generalizing to other grape varieties and wine styles. For instance, the relative aromaticity of the variety may mediate perception of LBT, and processing variables such as maceration technique, pressing regime and the use of thermo-vinification may also affect IPMP transfer from MALB and final concentration in the juice and wine.

Notwithstanding these qualifications, our results provide the first estimates of tolerance levels for MALB derived experimentally, and should prove a useful baseline for further trials. Encouragingly, these estimates are in general agreement with limits set by a number of wineries in Ontario and the USA based on their experiences over recent vintages. Further confidence in the data can be derived from the results of trial 1, where control Riesling wines could be differentiated from those made in the presence of the equivalent of 3000 beetles$\cdot t^{-1}$ grapes, but not when the density equivalent was 300 beetles$\cdot t^{-1}$, suggesting the threshold density lies between these two values.

Further considerations and research: As only ortho-nasal thresholds have been determined here, we encourage further research to establish retro-nasal sensitivity to LBT and to test our speculation that the former is the more sensitive mode for IPMP detection. In addition, determination of IPMP sensory thresholds for a range of wine styles, as well as consumer 'acceptance' thresholds for LBT would be useful. The importance of sensory evaluation in the assessment of LBT is obvious from this study, given that IPMP concentrations are generally below the limit of quantitation of the GC-MS system, yet LBT is observed in some of the wines. We recommend further development of techniques to increase the sensitivity of the analytical methods.

Given the uncertainties outlined above, it may be prudent for industry to adopt a more conservative tolerance level for MALB at harvest than the estimates derived from the trials described here. We recommend a MALB density of 200 - 400$\cdot t^{-1}$ as a likely 'safe' limit for wine-grapes to protect against the development of LBT in the subsequent wine. For illustration purposes, this equates to 800-1600 MALB per acre or one MALB for every 0.75 - 1.5 vines, assuming 4 tonnes per acre (0.4 ha) and 1200 vines per acre. This estimate could assist with acceptance/rejection decisions by wineries for grape harvesting and processing as well as quality-based remuneration schema, and with decision-making with respect to spray or other interventions in the vineyard. Accurate estimation of actual MALB density in the vineyard and harvested fruit may be challenging, and research is encouraged to develop robust sampling methods. Finally, these data should not be extrapolated to provide estimates of tolerance levels for grapes intended for juice production. Processing variables can differ to those employed in wine production (e.g. treatment at high temperatures), potentially influencing both MALB behavior and IPMP yields.

Conclusion

At the level of MALB addition examined here, LBT does not appear in finished wines if beetles are introduced and subsequently removed prior to crushing of the grapes. This result highlights the importance of subsequent processing (crush/destem, pressing and fermentation) in the extraction of IPMP and development of the taint. The 'tolerance limits' calculated here for MALB for the formation of LBT in the subsequent wines will likely be moderated by other grape and wine processing variables not considered in this study and by the specific grape variety. Thus we suggest that 200-400 beetles/tonne of grapes is a useful and likely conservative upper limit that should protect against LBT in wine. Taken overall, this information should assist with appropriate interventions in the vineyard/winery, in the setting of 'rejection' criteria for harvested fruit, and provide baseline targets for treatments aimed at reducing MALB density.

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