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Sensory detection thresholds of "ladybird taint" in 'Riesling' and 'Pinot Noir' under different fermentation and processing conditions

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Summary

The Asian ladybird beetles *Harmonia axyridis* feed on damaged fruits in late summer and in autumn, especially on grapes. By getting harvested and processed together with the grapes, they cause an off-flavor in the wine, the so-called "ladybird taint" (LBT). Depending on fermentation conditions of red wine variety 'Pinot Noir', panelists recognized the LBT at different concentrations: Fewer beetles were required in the non-heated treatment to elicit LBT. The sensory detection threshold for LBT in must fermented wines was about 3 beetles·kg^{-1} of grapes and in must heated wines it was about 6 beetles·kg^{-1} of grapes. In the white wine variety 'Riesling', the sensory detection threshold for LBT was at 4 beetles·kg^{-1} of grapes.

The main olfactory active compound causing LBT, 2-isopropyl-3-methoxypyrazine (IPMP), was detected by 50 % of panelists at a threshold of 1 ng·L^{-1} in 'Riesling' and 2 ng·L^{-1} in 'Pinot noir'. Thus, a threshold of five beetles with a specific amount of IPMP each in hemolymph processed within 1 kg of grapes can reach the human detection limit of 1-2 ng IPMP·L^{-1} of wine.

Modifications in wine processing conditions can reduce the LBT in wines. The mortality of beetles in the wine press can be reduced at pressures less than 2 bar and duration of pressing shorter than 60 minutes.

Key words: *Harmonia axyridis*, ladybird taint, wine processing, sensory detection threshold.

Introduction

Since 2001, the invasive species *Harmonia axyridis* has caused problems in temperate climate viticulture in the USA and Canada (Koch 2003). During the last years, this species has also started occurring in Central European vineyards (Kögel *et al.* 2010). *H. axyridis* is an extremely polyphagous predator of insects, but it also has a preference for ripened and injured fruits (Galvan *et al.* 2006). When *H. axyridis* feeds on grapes, it may be harvested and processed with the grapes, especially during mechanical harvesting. During processing the coccinellids release their hemolymph in the must by reflex bleeding or crushing. The hemolymph contains remarkable amounts of defensive substances, which can act against microorganisms (Gross *et al.* 2010) or as deterrents against natural enemies (Klausnitzer and Klausnitzer 1997). Additionally, some compounds may act intraspecifically as aggregation pheromones (Al Abassi *et al.* 1998). These substances cause a specific off-flavor in wine called "ladybird taint" (LBT). This flavor can be described as "bell pepper", "burned peanut butter" or "green vegetables" (Pickering *et al.* 2004, 2005). The LBT of wine was first described in the USA when millions of individuals of *H. axyridis* feeding within grapes were harvested. It is probably caused by several methoxypyrazines (Pickering *et al.* 2005, Galvan *et al.* 2008, Ross *et al*. 2010).

Methoxypyrazines are well known from wines processed from grapes of varieties like 'Cabernet Sauvignon', 'Merlot' or 'Sauvignon Blanc'. In typical German varieties like 'Riesling' or 'Pinot Noir' these substances are undesirable because of altering the typicity of wines (Pickering *et al.* 2005, Linder *et al*. 2009). Studies by Pickering *et al.* (2005, 2008 a) described 2-isopropyl-3-methoxypyrazine (IPMP) as the main component causing this off-flavor. In hemolymph of *H. axyridis* IPMP was detected with highest sniffing intensity compared to other methoxypyrazines (Kögel *et al.* 2012). According to Cai *et al*. (2007), the hemolymph of the invasive ladybird beetle contains IPMP in an average concentration of 0.16 ng·beetle^{-1}.

Pickering *et al.* (2007 b), Linder *et al*. (2009) and Galvan *et al*. (2007) determined or estimated sensory detection thresholds (SDT) of the LBT in several varieties. For 'Riesling', Pickering *et al.* (2007 b) extrapolated a SDT of around 1.5 beetles·kg^{-1} of grapes (that 75 % of test persons had detected) for blended wines (Control: LBT-wines). The LBT in 'Pinot Noir' was reviewed by Linder *et al.* (2009), estimating a SDT of 1 beetle·kg^{-1} of grapes.

Must fermentation is the traditional and desired way to process red wine. In Central Europe a second type of processing, the so-called must heating, is often used. The wine processing conditions which may affect the development of LBT during the first steps of processing have only been analyzed by a few publications. It has been found that reflex bleeding of live beetles had a lower impact on LBT than total crushing and death of beetles (Pickering *et al.* 2007 b, Galvan *et al.* 2007). Other factors that can influence the LBT or methoxypyrazines, respectively, in infested wine are as follows: the type of bottle closures (Pickering *et al.* 2010, 2009, Blake *et al.* 2009), the type of packaging and storage conditions (Pickering *et al.* 2009, Blake *et al.* 2009). Several yeast strains (Pickering *et al.* 2008 b)

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or treatments with oak chips or activated charcoal (PICKERING *et al.* 2006), may improve the taste of wine. PICKERING *et al.* (2007 b) announced that processing variables such as maceration technique, pressing regime or must heating may also affect IPMP transfer from the ladybird beetle into the wine. Until now, no further studies were conducted investigating the influence of these processing conditions on LBT in wine.

Therefore, in this study, both processing types (must heating and must fermentation) as well as pressing duration and pressure were compared with regards to their possible impact on LBT in wines from *H. axyridis* infested grapes. Also solutions for the reduction of LBT in wine at the beginning of processing are suggested and the sensory detection threshold of LBT in German wines spiked with methoxypyrazines and live adults of *H. axyridis* is specified. The sensory detection thresholds of methoxypyrazines and adult beetles for two common German wine varieties 'Riesling' and 'Pinot Noir' in dependence of different fermentation conditions are defined. Unlike previous studies, the data in this study were not extrapolated or estimated, but measured by adding live beetles to the grapes in different amounts.

Material and Methods

Insects: *H. axyridis* adults were collected during September 2009 in vineyards located close to the Julius Kühn-Institute in Siebeldingen. The beetles were kept at 9 °C in plastic jars in a refrigerator without food or water for one week. Twenty-four h prior to the experiments, the beetles were kept at 22 °C under daylight conditions.

Beetle mortality as a function of pressure: Nine adult *H. axyridis* were added to 3 kg of grapes ('Riesling'). The grapes were pressed in a pneumatic press (Type 6030, Willmes, Lorsch, Germany) at pressures between 1-5 bar. The variants at 2 and 5 bar were repeated 5 times to check the possible variation between the pressing procedures. Experiments at 1, 3 and 4 bar were repeated twice. All experiments were done using destemmed grapes, respectively. Beetles were collected out of the wine press by hand after pressing (15 min) and examined for mortality or damage. As ladybird beetles are known to feign death (thanatosis) when they have been disturbed, the individuals supposed dead checked again for locomotion activity for 24 h by aligning them upside-down in petri dishes. Those who had moved were considered alive. Those who stayed upside-down aligned were considered dead.

Beetle mortality as a function of pressing time: 15 kg 'Riesling' grapes were infested with 15 live beetles. The grapes were pressed for 90 min at 2 bar. After 30, 60 and 90 min the mash was mixed, the press stopped and the beetles inside the press were examined for mortality and injuries. Before continuing the pressing procedure, the predamaged beetles were reintroduced into the mash.

Processing 'Riesling' grapes infested with different numbers of beetles: In 2009, different numbers of adult beetles (0, 1, 2, 3, 4, 6, 8, or 20) were added to each 5 kg of 'Riesling' grapes (n = 2). Afterwards, grapes and beetles were pressed at 5 bar using a pneumatic press (Type 6030, Willmes). The must was filled in 5-L jugs, and sulphur dioxide was added to a final concentration of 50 mg·L^{-1}. The must was inoculated with yeast (Erbslöh Önoferm Freddo, 10 g·100 L^{-1}) for fermentation under room conditions (18-20 °C). After fermentation, an additional 100 mg·L^{-1} sulphur dioxide was added. After three weeks, the wine was separated from the yeast and decanted into several 0.5 L-bottles. Sparging with inert gas before bottling was not necessary due to the sensory assessment only two month later. The wines were tested by a sensory panel as described below (organoleptic testing procedure).

The trials were repeated in 2010 with reduced variants and amounts of grapes, but higher repetition numbers. 1 kg of 'Riesling' grapes were infested with 1, 5 and 10 adult beetles (n = 5). The grapes were pressed (at 5 bar) and the resulting must filled in 1 L-glass jugs. The further processing was the same as described above. The organoleptic properties of these wines were tested again by a sensory panel as described below.

Processing 'Pinot Noir' grapes infested with different numbers of beetles under different conditions: Due to the high numbers of live beetles necessary for each experiment, the trials were not repeated. Each 15 kg of 'Pinot Noir' were infested with 0 1, 2, 3, 4, or 10 adult beetles per kg of grapes, and transferred to 20-L-containers. In the first set of experiments, the must was fermented for 6 d (must fermentation), while in the second set of experiments the grapes were heated for 3 h in a water bath adjusted to 65 °C (must heating). Afterwards, the grapes were pressed with a pneumatic wine press (UVAmat, custom-made product, FH Geisenheim) at 2 bar and transferred to 15 L-glass bottles. After 3 weeks the wines were racked (Erbslöh Önoferm Freddo, 10 g·100 L^{-1}) and 100 mg·L^{-1} sulphur dioxide was added to the wine. The wines were tested by a sensory panel as described in the paragraph "organoleptic testing procedure".

Determining sensory detection thresholds of synthetic pyrazine IPMP in 'Riesling' and 'Pinot Noir' wine. A standard solution of 2-isopropyl-3-methoxypyrazine (IPMP) (Sigma Aldrich, Germany, 1 g/mL) was diluted three times in water-ethanol-solutions (10 µl per standard solution in 9 mL Milli-Q-water + 1 mL ethanol pA) to a final concentration of 10 ng·L^{-1} IPMP. Five bottles containing one year old 'Riesling' wine (filled in 2008) were inoculated with 2-isopropyl-3-methoxypyrazine (IPMP) in concentrations of 1, 2, 11, 50 and 100 ng·L^{-1}. Five bottles containing one year old 'Pinot Noir' were inoculated with IPMP in concentrations of 1, 3, 11, 50, and 100 ng·L^{-1}. The wines were tested by a sensory panel as described below.

Organoleptic testing procedure: A panel of ten persons (age 26-53 years; 2 females and 8 males; regularly winesensory trained) tasted in three tasting sessions (orthonasal and retronasal) the wines artificially infested with ladybird beetles (white wine from 2009 and 2010, and red wines from 2009) and the wines spiked with synthetic pyrazines (see above) in order to identify the special taste caused by methoxypyrazines or live beetles. The wines, presented in standard wine tasting glasses, were evaluated with the three-alternative forced choice (3-AFC) test in March 2010 and 2011 in the sensory evaluation room at Geilweilerhof, Siebeldingen under daylight conditions. Between the wine sets panelists were able to drink water and eat bread to neutralize the taste. Labeling of the uncovered glasses was done by numerical sets. The panelists were trained before the session with labeled control wine and labeled wine infested with the maximum number of beetles·kg^{-1} of grapes or the maximum amount of synthetic IPMP·L^{-1} in the trials. When 50 % of panel individuals had identified the altered taste, it was defined as sensory detection threshold (SDT; after definition of Bi and Ennis 1998). It could be read from the diagram at the point the quadratic function curve crosses the 50 %-threshold line.

Results

Beetle mortality as a function of pressure: An increase in pressure increased the mortality of *H. axyridis*. A pressure of 2 bar caused a mortality of 50 % (SD 5.6 %) of beetles. A reduction under 2 bar was not effective in reducing the mortality (still 50 %). At 3 bar, beetles were heavily crushed and the mortality increased to 60 %, at 4 bar 75 %. A maximum pressure of 5 bar resulted in 92 % (SD 5.8 %) mortality of the ladybird beetles (Fig. 1). The five-fold repetition showed that the pressing procedure in relation to the mortality of beetles was nearly constant (Fig. 1).

Beetle mortality as a function of pressing duration: A shorter pressing period reduced the mortality of beetles. It was 33 % after the first 30 min of the pressing and only a few beetles got crushed. After 60 min, the mortality of the beetles increased to 70 % and after 90 min the mortality rate was 96 % (Fig. 2). The trend shows a linear relation between mortality of beetles and pressing duration.

Sensory detection threshold (SDT) of 'Riesling' grapes spiked with different numbers of beetles: The SDT of LBT in 'Riesling' wines from 2009 and 2010 was similar. 6 beetles·kg^{-1}

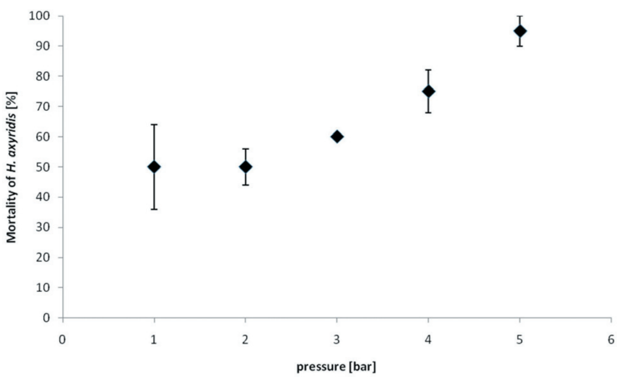

Fig.1: Beetle mortality [%] in a pneumatic press at different pressures [1-5 bar]. 5 kg grapes were pressed for 15 min. The means and standard deviations (n = 5) are given.

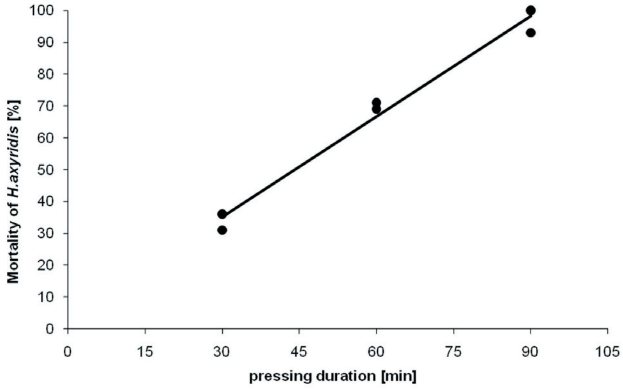

Fig. 2: Beetle mortality [%] after 30, 60 and 90 min in a pneumatic press at 2 bar in a two-fold repetition. Grapes and beetles were mixed every 30 min. A trend line is given.

of grapes were detected by 72 % of test individuals, while only 21 % detected 1 beetle/kg (trial 2009). In 2010 the LBT caused by 1 beetle·kg^{-1} of grapes was detected by 38 % of panelists. 52 % could detect 5 beetles·kg^{-1}, 89 % could detect 10 beetles·kg^{-1} and 100 % detected 20 beetles·kg^{-1} (Fig. 3). The mean of both years resulted in a SDT of LBT at 4 beetles·kg^{-1}.

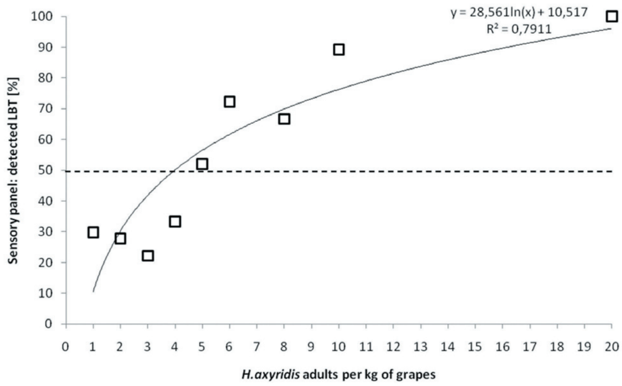

Fig. 3: Percentage of members of a sensory panel who detected "ladybird taint" (LBT) in 'Riesling' infested with different numbers of H. axyridis per kg of grapes in the years 2009 and 2010 (average of both years). The sensory detection threshold line is set at 50 %.

SDT of 'Pinot Noir' grapes infested with different numbers of beetles: The SDT of LBT in must fermented 'Pinot Noir' was lower in comparison to must heated wines using the same numbers of beetles per kg of grapes. This means that 3 beetles·kg^{-1} of grapes were detected by 50 % of panelists in must fermented wines. This threshold increased to 6 beetles·kg^{-1} of grapes when the grapes were must heated. The upper limit was also different: the must fermented wines infested with 10 beetles·kg^{-1} of grapes were detected by nearly 100 % of panel persons. Only 70 % of panel individuals detected LBT in must heated wines infested with same number of beetles (Fig. 4).

SDT of synthetic pyrazine in 'Riesling' and 'Pinot Noir' wine: The SDT of the synthetic methoxypyrazine IPMP determined for 'Riesling' was lower than for 'Pinot Noir'. In 'Pinot noir', 58 % of the panelists detected 2 ng IPMP·L^{-1}, while 51 % detected

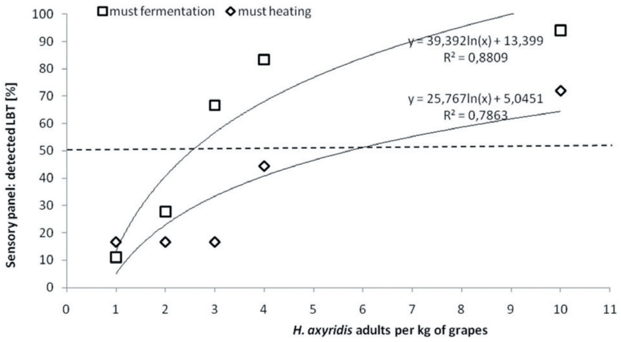

Fig. 4: Detected LBT in 'Pinot Noir' infested with different numbers of H. axyridis beetles per kg of grapes under different fermentation conditions (must heating vs. must fermentation). The sensory detection threshold line is set at 50 %.

as little as 1 ng IPMP·L^{-1} in 'Riesling'. 11 ng IPMP·L^{-1} or more were detected by all panelist in both varieties. 100 % detected already 3 ng IPMP·L^{-1} in 'Riesling'. 1 ng IPMP·L^{-1} in 'Riesling' was only detected by 10 % of panel persons (Fig. 5).

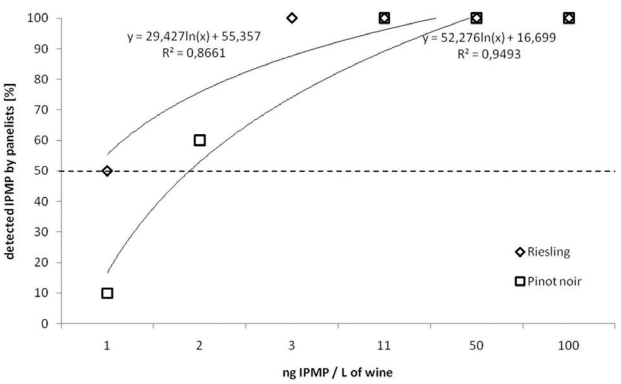

Fig. 5: Concentrations (1, 2, 11, 3, 50 and 100 ng·L^{-1}) of synthetic 2-isopropyl-3-methoxypyrazine (IPMP) in wine cultivars 'Riesling' and 'Pinot noir'detected by panelists [%]. The sensory detection threshold line is set at 50 %.

Discussion

Reducing the LBT by reducing the mortality of ladybird beetles: Crushing of ladybird beetles resulted in higher amounts of hemolymph in the must, while reflex bleeding alone does not affect LBT in a detectable way (PICKERING *et al.* 2004, GALVAN *et al.* 2007). Therefore, it is desirable that *H. axyridis* beetles do not get crushed during wine processing. Between 1 and 2 bar, no difference in mortality could be detected, but the number of crushed beetles increased at pressures higher than 2 bar. Additionally, pressing durations shorter than 60 min at 2 bar reduced the mortality of the beetles (Fig. 2). Pressing related damage may thus be responsible for their death and loss of hemolymph. The beetles may survive the pressing procedure for some time by hiding themselves interstitially between berries and stems.

Older wine presses like pipe presses and screw presses reaching pressures higher than 3 bar are rarer in big wineries. Modern wine presses like pneumatic presses reach only 1-2 bar. Lower pressures reduce tannin contents but also enable high must volume. The pressing duration is the second important point to optimize pressing procedures. Short time pressing is preferable in order to reach high must profits which increase economic efficiency and fast vintage. The average pressing duration for modern presses is mostly about 90 min. The aim of new pneumatic presses is a reduction of pressing power and duration – which coincides with the aim to reduce the amount of hemolymph of ladybird beetles in must.

After careful consideration of common wine processing conditions and the resulting mortality of beetles, it can be concluded that grapes highly infested with *H. axyridis* should be processed at very low pressure and over a short time with regard to grape quality. In order to obtain good wine tastes a reduction of must yield might be reasonable.

Sensory detection threshold (SDT) of LBT (number of beetles/kg of grapes or ng IPMP·l^{-1} wine, respectively) in 'Riesling' and 'Pinot Noir': For both the white wine variety 'Riesling' and the red wine variety 'Pinot Noir' (processed with must heating), the SDT was determined to be at 4 respective 6 beetles·kg^{-1} of grapes, as this number was detected by 50 % of the panel individuals (Fig. 4). In variants with 1-4 beetles·kg^{-1} of grapes, the LBT was more readily detected in 'Pinot noir' than in 'Riesling'. The SDT of LBT in 'Riesling' in this study is higher than the SDT of 'Riesling' extrapolated from data published by PICKERING *et al.* (2007 b). This might be due to a methodological difference between the studies as PICKERING *et al.* (2007 b) analyzed wine spiked with 3 beetles·kg^{-1} of grapes and diluted it in different amounts in a control-wine without beetles. As 75 % of panellists detected the blend 51:49 LBT-wine:control wine, PICKERING *et al.* (2007 b) calculated that 51 % of three beetles refer to 1.53 beetles·kg^{-1} of grapes. Our trials were carried out with beetles starved for one week prior to pressing procedure and a pressure of 5 bar. These facts may influence the SDT. For analysis of the red wine variety 'Red Bergamais' the same method was used with a second important difference between the studies, namely that the beetles were added to grape juice concentrate and not to the grapes. The extrapolated SDT resulted in 1.26 beetles·kg^{-1} of grapes. PICKERING *et al.* (2007) themselves cautioned that the estimated tolerance level for red wine could be less robust than for white wine because of different trial designs. But the data match the results of GALVAN *et al.* (2007), who estimated that the STD (50 % of panelists) was about 5 beetles·kg^{-1} of grapes. LINDER *et al.* (2009) confirmed these data for 'Chasselas' and 'Pinot noir' grape varieties.

The difference between our data and the SDT in 'Red Bergamais' calculated by PICKERING *et al.* (2007 b) could mainly be explained by the differing trial design: their wine was prepared by adding live beetles into a grape juice concentrate and not into the grapes before pressing, as seen in their trial design for 'Riesling' and in the presented study.

Thus, the SDT was only estimated by correlating the weight of grapes required to produce the grape juice concentrate with the number of *H. axyridis*/kg of grapes (Pickering *et al*. 2007 b). This should result in a higher SDT because the beetles were not crushed. Evidently, at a given pressure the SDT of LBT apparently depends on the number of beetles·kg^{-1} of grapes. A saturation curve is imaginable, indicating that the saturation is reached at an amount of maximum 20 beetles/kg of grapes (Figs 3 and 4).

Many studies describe 2-isopropyl-3-methoxypyrazine (IPMP) as main component causing LBT in wine (overview in Kögel *et al.* 2012, Pickering *et al.* 2005). Therefore, the difference in IPMP intensity was measured and calculated in two wine varieties, too. The sensory intensity of added IPMP in this study was 1 ng·L^{-1} in 'Pinot noir', and 2 ng·L^{-1} in 'Riesling' (Fig. 5). In 'Chardonnay', Pickering *et al.* (2007 a) obtained group best estimated thresholds at 0.32 ng·L^{-1}, for 'Gewuerztraminer' 1.56 ng·L^{-1}, and for a red wine blend at 1.03 ng·L^{-1} (all only orthonasal). Other studies estimated the threshold at 2 ng·L^{-1} for red wine (see in Sala *et al*. 2004) and for Sauvignon blanc (Allen *et al.* 1991). In two different types of grape juices ('Concord' and 'Niagara'), Pickering *et al*. (2007 a) detected an orthonasal threshold of IPMP between 0.74 and 1.11 ng·L^{-1}. In water, IPMP can be detected at a threshold of 2 ng·L^{-1} (Murray *et al.* 1970, Sala *et al.* 2005, Seifert *et al.* 1970). All these data assume that SDT is influenced by medium and in the case of wine by its style (see also Pickering *et al.* 2007 a). Finally, IPMP is detectable in all tested media at low levels between 0.32-2 ng·L^{-1}. Cai *et al.* (2007) determined an average IPMP content in the hemolymph of adult *H. axyridis* of 0.16 ng per adult beetle, but the contents varied between the specimens. Provided that from 1 kg of grapes in average 800 ml must could be processed, about 6 beetles·kg^{-1} of grapes would infest the resulting wine with about 1 ng·L^{-1} IPMP. This corresponds with the detection threshold for LBT in wine in this study. (Fig. 5).

In 'Pinot noir' wine the LBT was detected when at least 6 beetles had infested 1 kg of grapes, while a concentration of 2 ng·L^{-1} synthetic IPMP was detectable. These values may correspond when the beetles contain higher IPMP amounts than average. Further explanations for diverging SDTs in different studies might be due to annual differences in grape conditions, or the use of different methods during viticulture and viniculture, e. g. the use of different yeasts (Pickering *et al.* 2006, 2008b). Pickering *et al*. (2007a) reported, that detection thresholds for IPMP in grape juices 'Niagara' and 'Concord' were strongly influenced by grape variety, and concluded that the human detection threshold for IPMP in wine could be influenced significantly by wine style and evaluation mode. Grape varieties like 'Cabernet Sauvignon' or 'Sauvignon Blanc' contain pyrazines naturally in odour active amounts without any prior contact to ladybird beetles. In these grape varieties ripening, sun light exposure, irrigation or plantation density also influence methoxypyrazine contents (Hashizume *et al.* 1999; Sala *et al.* 2004, 2005). Therefore, a very wide range of methoxypyrazine levels can be found in grapes (Kotseridis *et al.* 1999). Normally 'Riesling' and 'Pinot Noir' have very little pyrazine contents but they can occur in low amounts depending on grape growth conditions. Perhaps 'Pinot Noir' used in this trial had naturally less IPMP content than 'Riesling'. We are currently conducting studies to evaluate several factors which may influence the methoxypyrazine contents of *H. axyridis*.

Reducing the LBT by using different types of fermentation: In Central Europe two types of wine processing, the must fermentation and must heating, are commonly used in vinification. Must heating is mainly used for red grapes contaminated with grey mold caused by *Botrytis cinerea*. As compared to other wine growing regions, this approach is mostly used in cool climate viticulture (Jakob 1980). Must fermentation is the classic way to get the red colour out of grape skin and into must. Common duration of fermentation is between two to 30 d depending on fermentation temperature and deliberate wine style (Dippel 2000). We calculated a SDT in must heated 'Pinot Noir' at 6 beetles·kg^{-1}. The SDT in 'Pinot Noir' decreased to 3 beetles·kg^{-1} when the must had been fermented (Figs 3 and 4).

After one day of maceration, Sala *et al.* (2004) recognized an increase of natural IBMP content in grapes of 'Cabernet sauvignon', but after racking, no further increase was observed. The present results suggest that there was an increase in IPMP during maceration when grapes are infested by *H. axyridis.* This could be due to the high extraction of pyrazines, the probable main compounds responsible for the LBT (Pickering *et al*. 2005; Galvan *et al*. 2008), in alcohol. In contrast, the contact to alcohol is very low in the must heated variant. There might be a loss of methoxypyrazines by warming in the must-heated experiment and therefore possible volatilization of pyrazines. Therefore, the type of fermentation used in previous studies also plays an important role and may have resulted in the wrong conclusion that red wines generally had lower SDT than white wines. Linder *et al*. 2009 observed a lower SDT in must fermented red wine 'Pinot noir', than in non-must fermented white wine variety 'Chasselas'. Also Pickering *et al.* (2007 b) used must-fermentation for the grape juice concentrate of 'Red Bergamais' instead of no must-fermentation for the white wine variant.

Finally we can conclude that the fermentation type influenced the LBT which could be reduced by using the must heating technique instead of must fermentation during vinification. A short-term analysis is necessary which will take into consideration the common harvest and processing conditions with regard to higher economic efficiency and lower LBT.

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