FREE AND BOUND VOLATILES IN WINES AND GRAPES: ANALYSIS, EFFECTS OF WINEMAKING AND GRAPE GROWING, AND THEIR CONTRIBUTION TO WINE QUALITY

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FREE AND BOUND VOLATILES IN WINES AND GRAPES: ANALYSIS, EFFECTS OF WINEMAKING AND GRAPE GROWING, AND THEIR CONTRIBUTION TO WINE QUALITY

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The aroma of wine is due both to volatiles present in the grapes ("free") as well as non-odorous ("bound") precursors that are released during fermentation. 3-alkyl-2-methoxypyrazines (MPs) are a well known class of free volatiles possessing an herbaceous odor. Because high concentrations of MPs can negatively impact wine quality, there is a considerable interest in reducing MP concentrations, but current enological practices lack selectivity. Unlike MPs, most wine aroma compounds exist in grapes as bound forms. Treating juices with silicone prior to fermentation resulted in a reduction of MPs by 53 to 93% without affecting the majority of other volatiles in finished wines. We also considered factors that would influence MPs during the growing season. In the field, MPs decrease during ripening, but the degradation pathway is not well defined. We hypothesized that the 3-isobutyl-2-methoxypyrazine (IBMP) is degraded to its hydroxyl form (IBHP), based on an inverse correlation of the two compounds in bell peppers (R^2=0.958) and Cabernet Franc grapes (R^2=0.998) during ripening. Most key odorants in wine cannot be detected in grapes because they exist in bound forms such as glycosides. The behavior of glycosides during ripening is still not well understood, even though this

knowledge is important for determining optimum harvest time and providing insights into grape biochemistry. Following acid hydrolysis to release the aroma from the bound precursors, we tracked the behavior of three major glycoside classes during grape maturation in 2009 and 2010 seasons. Monoterpene and C_{13} norisoprenoid glycosides both increased during maturation, but monoterpene glycoside accumulation occurred ~2 weeks later. The behavior of benzenoid glycosides was inconsistent. Hedging – a common viticultural practice – resulted in no significant impact on glycosides at harvest.

BIOGRAPHICAL SKETCH

Born and raised in Sumatra, Indonesia, Imelda Ryona had come all the way to the United States of America to pursue a better education. Following her passion for food and science, she started her educational journey majoring in Food Science and Technology at California Poly Technique University (Cal Poly) in Pomona, CA. After she completed her Bachelor of Science degree, she joined Cornell University's Department of Food Science as a summer scholar working under Dr. Olga Padilla-Zakour. Having no experience in research, she then discovered her great interest in that field. With Dr. Padilla-Zakour's encouragement and support, she applied to Cornell graduate school after completing the 3-month summer research in 2006. From spring 2007, she was admitted as a Master of Science graduate student at the Department of Food Science working under Dr. Gavin L. Sacks. The story at Cornell did not just evolve around her educational journey. In the year working as a summer scholar, she met Johannes Reinhardt who was and is a winemaker at one of the wineries in the Finger Lakes. Even though she never had planned to stay longer than 3 months as a summer scholar in upstate New York, she ended up settling here by continuing her graduate commitment as a Ph.D. student and marrying Johannes Reinhardt in 2008. To Imelda Ryona and perhaps many others as well, Cornell University is not just a great place for education but can also be a great place to meet the love of one's life and start a family.

I dedicate this accomplishment to my beloved husband, Johannes Reinhardt and my beloved best friend, Friska Nataline Silitonga.

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Without a great deal of support from many wonderful people, I would not have been able to accomplish the work written in this dissertation. With my heartfelt appreciation, I would like to acknowledge the following people: Dr. Gavin Sacks, my major advisor, for his intellectual contribution, enthusiasm, and respectful advising skill; Dr. Olga Padilla-Zakour, my committee member, for giving me the golden opportunity at Cornell through the summer scholar program and her nurturing character; Dr. Terry Acree, for always making me feel good and calm; Debby Ditzell and Janette Robbins, the administrative clerks, for always being so sincerely helpful; Tom Gibson, Herb Cooley, John Churey, the technicians, for being so resourceful and helpful; Roby and Imelia Chandra, my beloved parents, for loving and supporting me so purely and equipping me with great values for life; Sue and Kusnadi, my siblings, for believing in me; Elfriede and Otmar Reinhardt, Caroline and Walter Volkamer, my in-laws, for believing in me.

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LIST OF ABBREVIATION

3-alkyl-2-hydroxypyrazines (HPs)

3-alkyl-2-methoxypyrazines (MPs)

3-isobutyl-2-methoxypyraine (IBMP)

3-isopropyl-2-methoxypyrazine (IPMP)

3-*sec*-butyl-2-methoxypyrazine (SBMP)

3-ethyl-2-methoxypyrazine (ETMP)

3-isopropyl-2-ethoxypyrazine (IPEP)

Headspace solid phase microextraction (HS-SPME)

Solid phase extraction (SPE)

Liquid extraction (LE)

Gas Chromatography Mass Spectrometry (GC-MS)

Comprehensive 2 dimensional Gas Chromatography Time of Flight Mass Spectrometry (GCxGC-TOF-MS)

Multidimensional Gas Chromatography olfactory Mass Spectrometry (MDGC-O-MS)

Chapter 1

The 3-alkyl-2-methoxypyrazines: Sensory Studies, Biochemical Pathway, and the Insights into the Use of HS-SPME Analytical Technique for Achieving Accurate and Precise Quantifications in Grape Juice and Wine

Introduction

The 3-alkyl-2-methoxypyrazines (MPs) are widely found in the plant kingdom (*1*), in a wide variety of food products (*2, 3*), and even in insects such as multicolor Asian lady bugs (MALB) (*4*). Their presence can be formed by thermal treatment or by microorganism or can be naturally occurring (*2*). In thermally processed foods such as coffee (*5, 6*), roasted peanuts (*7*), cocoa (*8*), and beer (*9*), the presence of MPs is formed by maillard reaction via Strecker degradation. In products such as drinking water (*10*), cheese and milk (*11*), and apple juice (*12*), the presence of MPs is undesirable and is formed by microorganisms. In a wide variety of vegetables such as bell peppers, peas, potatoes, beets and carrots, MPs are synthesized in situ and impart a vegetative or herbaceous odorant (*1*). Each type of vegetable was dominated by different abundance of specific MPs. For instance, beet root and carrot are dominated by 3-*sec*butyl-2-methoxypyrazine (SBMP), bell pepper has the most abundance of 3-isobutyl-2-methoxpyrazine (IBMP), and pea is dominated by 3-isopropyl-2-methoxypyrazine (IPMP). As shown in Figure 1, these 3 types of MPs are also the most widely studied MPs in wine grapes. Similar to plant, MPs in wine grapes are synthesized in grapes. IBMP is the

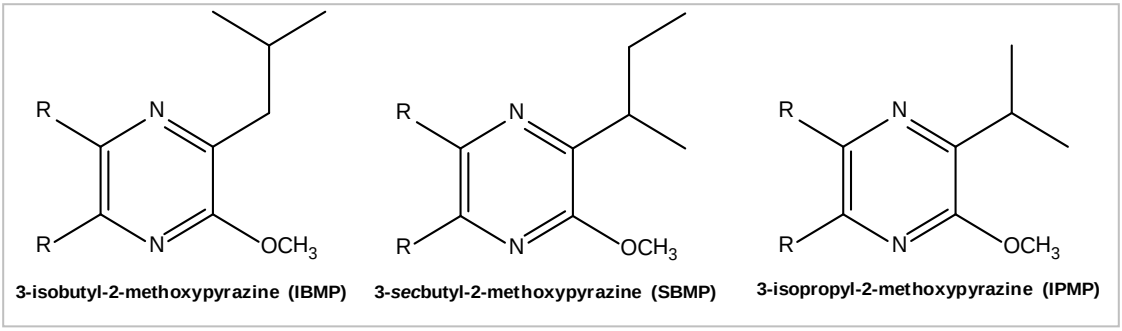

Figure 1 - The three most studied MPs in wine grapes and wines

most dominant MPs followed by IPMP which typically accounts for 10% of IBMP concentration (*13, 14*) unless an infestation of MALB in the grapevine is noted. In the field, the abundance of IBMP has been clearly demonstrated to be dependent on cultivar, climate, fruit maturity, and other viticultural practices on the vines that impact grapevine growing conditions (*15-20*). In wine grapes, most studies focused on IBMP. Thus, review of IBMP has been reserved in Chapter 2 and 3 while the brief reviews of the minor MPs are cited below.

IPMP in wine grapes and wines

The presence of IPMP in wine grapes and wines is usually less than IBMP unless infestation of MALB – which usually starts around harvest – is encountered. The MALB was intentionally brought to North America in the 1980s to control aphids (*21, 22*). Unfortunately, the intended remediation of aphid control had led to a problem in the wine industry. The MALB species that had been investigated to contain IPMP are the *Harmonia axyridis,* Hippodamia convergens, and Coccinella septempunctata (*23*). Per one *H. axyridis* lady beetle, the levels of MPs had been reported to be at 27.5 µg

(IPMP), 2.6 µg (SBMP), and 3.2 µg (IBMP). In a series of three-alternative forced choice (3-AFC) tests, it suggested that 10% of the population could detect the herbaceous off-flavor at a rate of 1.9 *H. axyridis* beetle per kg of *Vitis vinifera* Frontenac (*24*). Although both alive and dead MALB are capable of secreting MPs, the dead one secreted significantly less IPMP (*23, 25, 26*).

SBMP in wine grapes and wines

Observing the value of IBMP, IPMP, and SBMP in vegetables, SBMP represents the least amount compared to the other 2 MPs (*1*). In wine grapes, concentration of SBMP is usually less than 1 ng/L and typically account for 2% of IBMP concentration (*14, 27*). Thus, SBMP is the least studied MPs in wine grapes and wines.

ETMP in wine grapes and wines

Most studies had focused on the other 3 MPs. There were only a few studies reporting the detection of the 3-ethyl-2-methoxpyrazine (ETMP) in grapes. In vegetables, ETMP had been reported as the key odorant in cooked potatoes (*28*) and its sensory detection threshold is much higher than the other 3 MPs (425 ng/L in water) (*29, 30*). Typically, ETMP is detected at a level below its sensory detection threshold (*16*). For instance, Pinot noir, known as a variety that does not exhibit the unpleasant bell pepper or herbaceous aroma, had shown to have 100 ng/L. Cabernet Sauvignon had a much higher level greater than1000 ng/L (*29*). Although ETMP was reported to increase sharply toward

the end of the ripening stage (*31*), it showed no clear relationship to either fruit ripening or fruit sunlight exposure (*16*).

Sensorial evaluation of MPs in wines

Sensory detection threshold of MPs is very low and varies depending on matrices. For instance, IBMP detection thresholds in water, model wine, and white wine were reported to be at 0.5 to 1 ng/L (*13, 32*), 2 to 6 ng/L (*33, 34*), and 2 ng/L (*13*) respectively. Compared to IBMP, the detection threshold of IPMP is lower, reported at 0.32 ng/L in a non-aromatic Chardonnay white wine (*35*). In red wine, the detection threshold of IBMP is usually higher than in non-aromatic white wines having around 10 ng/L to 16 ng/L (*32-34*). This is due to a more complex aroma bouquet in red wine than the non-aromatic white wine. Between 8-15 ng/L, the presence of IBMP in wine is considered to impart a positive aroma characteristic while above 30 ng/L is overwhelmingly unpleasant and considered as a fault to wine aroma bouquet (*29*). Sensory evaluations employing descriptive analysis have always reported a consistent observation showing vegetal attribute and fruity attribute on the opposite end of the vector on a plotted principal component analysis (PCA) (*36-39*). This suggested that these 2 aroma attributes are inversely correlated - as the intensity of fruity aroma increases, the vegetal aroma decreases or vice versa. The presence of MPs in wines has been widely reported to be correlated to the presence of vegetal aroma in wines. However, they are not linearly correlated (*33, 39*). This suggested that MPs were not the only group of aroma

compounds that contributed herbaceous aromas in wine. Besides the fact that the presence of fruity aroma compounds can suppress or reduce the intensity of vegetal note, Escudero et al. had demonstrated that other green-smelling compounds such as 1-hexanol and *(Z)*-3-hexenol had played a role in their contributions to herbaceous aroma in wines (*40*).

Biochemistry of MPs in grapes

Involving a series of chemical reactions – amidation, condensation, and O-methylation shown in Figure 2, a putative biosynthetic pathway for the MPs in plants was first proposed over 40 years ago (*28, 41*). The initial step of synthesis started with either α-amino acids of leucine, valine, or isoleucine to form a final product of IBMP, IPMP, and SBMP respectively. The next step involved in amidation of the amino acid to form is amide form.

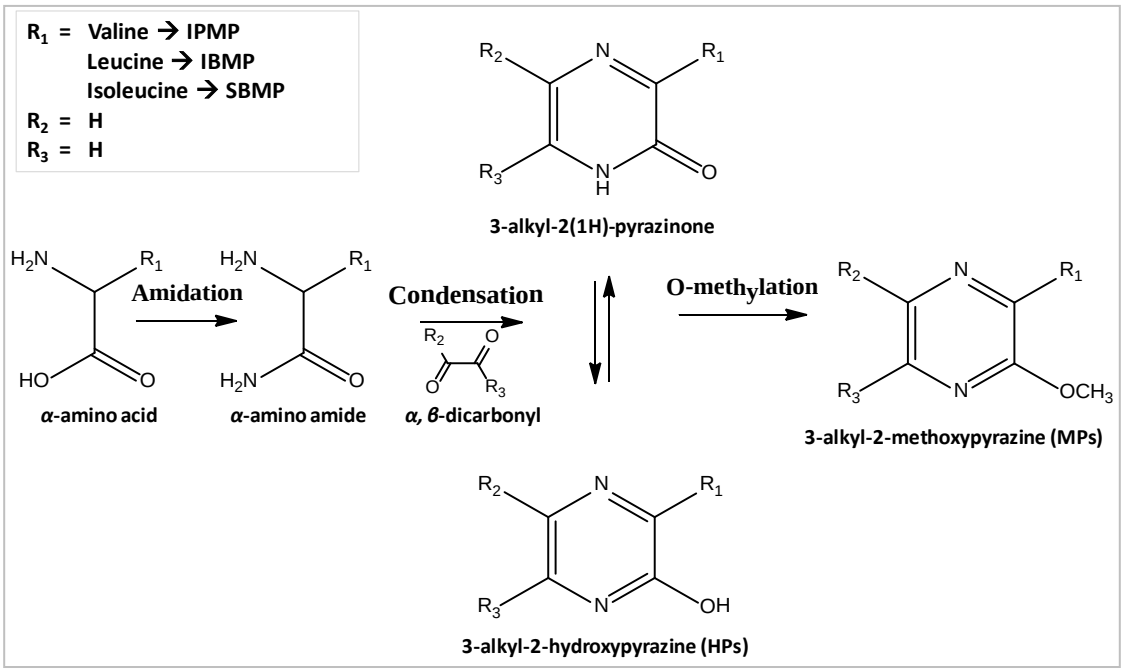

Figure 2 - Biosynthesis pathway of MPs

Then, a step of pyrazine ring formation was proposed through condensation of the α-amino amide by an *α,β*-dicarbonyl species from either a glyoxal or glyoxylic acid (*1, 28, 41*). Since there is no biochemical evidence that an α-amino acid can form its corresponding amide (*28*), Murray et al. suggested that the source of the second nitrogen for amide formation could be derived, apart from the proposed amidation, from a mono-nitrogen dicarbonyl species or a direct involvement of ammonia (*1*). To date, neither α-amino acid amide nor glyoxal had been isolated from grape tissue. Thus, this first step of biosynthesis is yet to be proven. The last step of this putative biosynthesis involved an *O*-methyltransferase (OMT) enzyme to convert the 3-alkyl-2-hydroxypyrazines (HPs) into the methoxypyrazine form. The OMT, *S*-adenosyl-L-methionine (SAM) dependent methyltransferase, had been detected in a crude extract of Cabernet sauvignon grapes and proven to have the capability of methylating HPs to form MPs (*42*). Subsequently, the extract was further purified, characterized, and sequenced to produce 20 amino acids at the N-terminal (*43*). In 2010, a group in Australia used this partial N-terminal sequence to search the grape vine genomic sequence and was able to identify and cloned 2 putative *O*-methyltransferases (*VvOMT1* and *VvOMT2*) genes that are capable of forming MPs via *O*-methylation of HPs. The group observed a lesser catalytic efficiency *VvOMT2* (*44*). Recently, structural and functional relationships of these 2 genes isolated from *Vitis vinifera* Carmenere had been studied and had observed a sterical hindrance in *VvOMT2* thus

decreasing its catalytic efficiency (*45*). Although the recent work validated the final step of MP synthesis, in which part of grape vine organs that MPs were synthesized still remained unknown. Observing a highest fraction of IBMP in leaves compared to other parts of grape organs, the synthesis of MPs had been suggested to take place in the leaves followed by transportation into the grapes (*46*). However, a recent inter-varietal grafting experiment between Muscat and Cabernet Sauvignon had observed that MP cannot be translocated from the leaves to the berries as IBMP could not be detected (< ng/L) on Muscat clusters that were grafted on Cabernet Sauvignon vines (*47*). Although MPs are known to decrease markedly during ripening - between veraison and harvest – in wine grapes (*20, 29*), no study has ever investigated the possible MP degradation product until recently when Ryona et al. reported a strong inverse correlation between MP degradation and HP accumulation in both bell pepper and wine grapes during ripening (*48*). The group proposed that MPs might be degraded to re-form its precursor HPs, thus reversing its final biosynthesis pathway. Whether this decrease is enzymatic or non-enzymatic, it is yet to be established.

Analytical technique for MP quantification in wine grapes and wines

MPs analysis and challenges

The presence of MPs in plant was first reported in and characterized as the key aroma in bell pepper (*49*). MP content in green bell pepper is at the range

of µg/L which is more than 100 times higher than the level in wine grapes and wines (ng/L). At such a low level, analysis of MPs had been challenging especially in wine, a much more complex matrix than wine grape. To accurately and precisely quantify MPs in grapes and wines, one needs effective pre- or on-column separation techniques and a highly sensitive detector to be able to detect the trace level MPs. Prior to the advancement of analytical tools and techniques such as head space solid phase microextraction (HS-SPME) and GC-MS, initial attempts of IBMP quantification in Cabernet Sauvignon (*50, 51*) and Sauvignon blanc (*52*) were unsuccessful. Subsequently, a laborious sample preparation involving steam distillation followed by solid phase extraction was introduced to quantify MPs reporting a very high detection limit (1.2 µg/L) with a large sample volume (500 mL wine) and a very poor recovery - *ca.* 50% (*53*). A less laborious method was introduced; however, it required a specialized apparatus and still reported a high limit of detection, 0.5 µg/L (*54*). Using a deuterated isotope internal standard and a selective MP extraction method (strong ion exchange), another group reported a much higher sensitivity of detection limit at 0.1 ng/L (*55*). The use of deuterated labeled internal standard not only facilitates a correct identification but also improves accuracy in quantification. After the introduction of HS-SPME technique (*56*), most studies on MPs have used this technique because of its fast, automated, and solvent-less features (*18, 47, 57-62*).

Headspace microextraction (HS-SPME) technique

Besides its strength on the automation, fast, and solvent free features, HS-SPME also provides a very high concentration factor *ca.* 3000X. Thus, it is very suitable for MP extraction technique as it amplifies the signal intensity of low concentration MPs. Despite all these strengths, care should be taken when employing this technique especially due to 1) unresolved co-elution, 2) a poor precision without the use of internal standard, and 3) matrices. In HS-SPME technique, no sample clean up is introduced. The selectivity of this technique relies mainly on its extraction choices, which is featured by various types of fiber coatings and sampling techniques. Various fiber coatings provide extraction selectivity over physical and chemical properties such as molecular weight and polarity of the compounds. Sampling techniques are either headspace or direct immersion of liquid samplings for extracting high volatile and less volatile to non-volatile compounds respectively. As mentioned previously, wine aroma bouquet is very complex containing more than 1000 volatiles of small molecules with a wide range of polarity and intensity. Without a pre-column sample clean-up, a complete resolution for quantification of low concentration analytes such as MPs could be very challenging. Studies had demonstrated that an additional separation step using 2-dimensional GC is warranted to resolve the co-elution problem (*4, 62, 63*). Most fiber phases, except the single-phase PDMS, have limited capacity and extract compounds based on the adsorbent binding mechanism where analytes compete with each other for binding sites. The single phase PDMS has a larger extraction

capacity and an absorbent binding mechanism which does not create competition among analytes. Comparing single phase PDMS to other types of fiber coatings, studies have shown a lesser efficiency in PDMS for extracting MP than other types especially the 2-cm 3-phase fiber (*58, 64*). Because of the competition on the binding mechanism, HS-SPME extraction technique is prone to a poor precision if no internal standard is used to correct the variation from run to run. This had been demonstrated by a study where 5 replicates of calibration curve ranging from 7 to 70 ng/L showed an average RSD of 39% (ranging from 32% to 55%) without the use of an internal standard (*64*). It's very critical to learn that the precision of HS-SPME technique is very poor without the use of an internal standard, particularly the isotopic labeled internal standard as demonstrated

Table 1 – A better precision in MP analysis with the use of an isotopic labeled internal standard in a HS-SPME technique

	Peak area		**Ratio**	**Overall RSD for different replications by:**		
Replicates	**m/z 124**	***m/z* 127**	**124/127**	**Area-m/z 124**	**Area-*m/z* 127**	**Ratio**
1	182416	437411	0.417	**32.6%**	**34.1%**	**5.2%**
2	154315	375394	0.411			
3	152848	379455	0.403			
4	117179	257226	0.456			
5	105984	237958	0.445			
6	71388	159183	0.448			
7	73738	186467	0.395			
8	106135	258522	0.411			
9	92448	225583	0.410			

Source: Koch et al. 2010 (*47*), with the corresponding author's and publisher permissions

by Koch et al. (*47*). We had acquired the permission to reuse their data as

shown in Table 1. From a same pool of samples differing in the GC runs, the RSD of peak area was *ca.* 30%. The signal intensity of *m/z* 124 by peak area ranged from 71388 to 182416 while the signal of *m/z* 126 ranged from 186467 to 437411. When an isotopic labeled internal standard was used, the variation of signal intensity among GC runs was corrected by dividing the analyte peak area to internal standard peak area. The result showed a RSD of only 5.2%. The use of an isotopic labeled internal standard will not only result in high precision but also accurate analyses. When a non-isotopic labeled internal standard is used, precision, not the accuracy, of the analysis could be improved. Thus, a critical evaluation should be employed to acquire results from any MP studies that use peak area or peak ratio with a non isotopic labeled internal standard for quantification. Grape juice and wine have a very different volatile composition. Ethanol is the major difference between the 2 matrices. Because of the competition on the fiber binding site, the presence of ethanol in wine had reduced the efficiency of MP extraction. This matrix difference should be noted as studies had reported an increase of signal sensitivity as the level of ethanol decreased (*26, 58, 64*). Among varietals, especially red and white wines, the volatile compositions are very different and pose a concern on matrix differences. In HS-SPME analysis, there are many parameters such as pH, extraction temperature and time, and types of fiber can affect the signal of the extracted analytes. Thus, many studies had been conducted to optimize the condition of these parameters for MP analysis. Some studies proposed a single factor optimization (*26, 57, 58, 64*) while

others proposed multivariate studies for a multiple factors optimization (*60, 62, 65*). As demonstrated earlier, critical evaluation should be employed to extract results from these optimization studies where peak area was used for analysis. Finally, when employing HS-SPME technique for MP analysis, it's recommended to 1) use an isotopic labeled internal standard, 2) use of the isotopic labeled internal standard coupled to 2 dimensional GC if analyzing wine samples. If a two-dimensional GC is not available, one can do a pre-column sample clean-up such as the use of solid phase extraction. However, this step does not guarantee a complete resolution from any interference in some cases. On the following sections, there are examples from studies reporting failure in resolving co-eluting peaks using a one-dimensional GC.

Solid phase Extraction SPE

Sample preparation is vital for selectively isolating desired analyte(s) from complex matrices and greatly influences the reliability and accuracy of analysis. Correct sample preparation can reduce analysis time, sources of error, enhance sensitivity, and enable unequivocal identification, confirmation, and quantification. There are many different types of SPE sorbent materials such as one mode polymeric or a mixed mode combining polymeric and ion-exchange resins. Polymeric and mixed mode resins with cationic or anionic exchange highlighting varied parameters in particle size, functional group, surface area, pore area and pore volume (*66*). SPE is a type of sample

preparation techniques that had been used for MP analysis. Studies have shown that this sample preparation technique did not resolve the interference issue. For instance, firstly, Lacey et al. extracting MP using a strong ion exchange resin reported failure to quantify IPMP in wine matrix due to co-elution (*14*). Secondly, using lichrolut EN - a stytrene-divinylbenzene polymer, 2 studies showed a different result even though they used the exact SPE procedure coupled to MDGC-MS for MPs quantification in wine and must. One failed to separate the interferences from the labeled internal standard, *d3*-IBMP and IBMP (*63*) and yet the other study showed a successful separation using a surrogated internal standard (*67*). This might be accounted for the matrix differences. Thirdly, using SPE – Strata SDB-L resin with a one-dimensional GC-MS, a study reported a high level of IBMP (1400 ng/L) in *Vitis vinifera* Primitivo red wine (*68*). Since most studies had never reported such a high concentration of MP in wine, and this variety has never been reported for possessing herbaceous aroma, it is very likely that this level of MP has been over estimated in a one-dimensional GC-MS analysis. Furthermore, the SPE resin (Strata SDB-L) used in this study has a similar functional (stytrene-divinylbenzene polymer) as that of Lichrolut EN which is a type of resin that has failed to selectively isolate MPs from interferences (*63*).

Conclusion

For analysis of MP using HS-SPME, the use of an isotopic labeled internal standard is a must for precise and accurate quantification. For quantification at

the level below 20 ng/L, a two-dimensional GC is warranted to ensure a complete resolution. Alternatively, pre-column sample clean-up coupled to HS-SPME analysis can be performed for analysis using only one-dimensional GC.

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Chapter 2

Pre-fermentation Treatment of Grape Must with Silicone to Selectively Reduce 3-Alkyl-2-methoxypyrazine Concentrations in Resulting Wines

Introduction

The potent, herbaceous-smelling 3-alkyl-2-methoxypyrazines (MPs), and particularly 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (SBMP), are widely distributed in the plant kingdom (*1*). Several references to MPs in the literature have focused on their role in wines and grapes (*2, 3*), in part because these compounds can mask fruity aromas desired by many wine consumers (*4*). IBMP and IPMP can exist at or above their sensory thresholds in wines, 8-15 pg/mL and 0.5-2 pg/mL, respectively (*5*-*7*). MPs in wines can originate from grapes, especially those in the Cabernet family (*8*), but they may also be introduced through exogenous contamination of grapes by the multicolored Asian lady beetle (MALB, *Harmonia axyridis*) (*9*). Considerable efforts have been made to identify viticultural and enological treatments to selectively reduce wine MP concentrations. Of these, viticultural strategies have generally been more effective. For example, cluster exposure to sunlight will inhibit IBMP accumulation pre-veraison (*10, 11*) while conditions that increase vine growth, including irrigation (*12*) and low crop loads (*13*), are correlated with higher MPs in grapes at harvest. Even with appropriate practices, excessive MPs in harvested grapes may still occur due to an

insufficient ripening period or MALB contamination. Post-harvest treatments to remediate MPs have generally been ineffective or suffered from a lack of selectivity and are summarized in Table 1. MPs are predominantly located in the skins (*14*) and are relatively stable during vinification, such that MPs in wines are well correlated with MPs in grapes when conventional red winemaking practices are used (*15*). Clarification of must prior to fermentation reportedly reduces IBMP by about 50% (*14*), but this practice is not suitable for red wine production and potentially would still leave an unacceptable concentration of MPs. Thermovinification is reported to reduce MPs, putatively by evaporative losses of MPs from the must (*16*), but this process results in clear sensorial changes to the finished wine (*17*). Yeast strain selection as well as post-fermentation cellar practices like bentonite fining and oak chip additions did not reduce MPs, although the latter can reduce perception of MPs due to masking (*18*). Activated charcoal fining (*18*), as well as some packaging materials (*19*), have been reported to reduce MPs in wines, but these approaches are expected to result in non-selective losses of other odorants. Because MPs, unlike the majority of aroma compounds in finished wines, are present in must before fermentation ("primary odorants") (*3*), we reasoned that pre-fermentation treatments of must to remove MPs should be more selective than post-fermentation treatments. We chose not to investigate activated charcoal: although this material will remove MPs, charcoal is also capable of binding and removing desirable semi-polar compounds, e.g., anthocyanins, and potentially some aroma precursors.

Alternatively, highly non-polar materials should be able to absorb non-polar MPs without removing semi-polar compounds. The log P of IBMP is reportedly 2.44±0.09 (*20*), and the estimated log P for IPMP is 2.41±0.38 (Advanced Chemistry Development Software V11.02, Ontario, Canada). Silicone (polydimethylsiloxane, PDMS) is non-polar, inert, available in food-grade forms, and widely used in food and pharmaceutical products (*21, 22*). The capacity of silicone to absorb MPs is well established through its use as a coating on solid-phase microextraction (SPME) fibers (*23*). The ability of polymers, particularly polyethylene, to absorb ("scalp") aroma compounds from foodstuffs is well known (*24-26*), although the majority of publications have focused on unwanted losses, e.g., the loss of monoterpenes from orange juice, rather than removal of undesirable compounds. In wine, polyethylene in the form of plastic kitchen wrap can reportedly be used to remove the highly non-polar 2,4,6-trichloroanisole (TCA, 'cork taint' aroma, log P =3.95) (*27*). Synthetic corks have been reported to scalp MPs as well as several other compounds from wine (*19, 28-30*). Outside of wine, polymers such as cellulose acetate, polyamide (*31, 32*), and divinylbenzene-polystyrene (DVB/PS) (*31, 33*) have been widely studied in the citrus juice industry for debittering. These polymers are somewhat polar, and DVB/PS in particular is known to effectively absorb not only non-polar analytes but also polar ones such as anthocyanins and many glycosylated precursors (*34*), and thus would not be appropriate for treatment of juice.

Table 1 - Summary of existing literature concerning removal of MPs from grapes or wine

Reference	Matrix	MPs studied[1]	Technique	% reduction	Caveats
This report	Grape juice	B, P	Silicone addition to juice/must	50-90%	Not appropriate for finished wines; only for musts
(*18, 35*)	Wine	B, S, P	Fluorescent & UV lights	n.s.	Ineffective
(*28*)	Wine	B, S, P	Addition of synthetic closures to wine	70-89%	Lack of selectivity
(*19*)	Wine	B, S, P	Packaging - Tetrapak carton	26-45%	Lack of selectivity, demands specialized packaging operations
(*36*)	Grape juice	P	Yeast selection	n.s.	Ineffective
(*18*)	Lady beetle tainted Wine	P	Activated charcoal	34%	Lack of selectivity
		P	deodorized oak chips	6%	Lack of selectivity
		P	Bentonite and oak chips	n.s.	Oak aromas may be inappropriate
(*14*)	Grape juice	B	24 hr must clarification (200 NTU)	50%	Not appropriate for skin-fermented wines
(*23*)	Model juice	S, B, P	Oak sawdust, gallic acid, epicatechin	n.s.	Ineffective
(*16*)	Grape juice	B	Thermovinification (60 ºC)	29-67%	Produces cooked aromas(*)
(*37*)	Grape stems	B	Steam treatment at 100 ºC/ 60 min	>95%	Not designed for juice/must

[1] B: IBMP, S: sBMP, P: IPMP *(*38*)

Polyethylene treatment of must could potentially be effective, but in preliminary trials in our lab, higher loadings of polyethylene film led to noticeable plastic off aromas. In this report, we evaluated the effectiveness of pre-fermentation silicone treatments on juices or musts to selectively reduce MPs in wines. A wide range of fermentation parameters were investigated, including fermentation temperature, skin contact, grape variety, and fermentation size.

Materials and methods

Chemical materials

Dichloromethane (DCM) and methanol were purchased from Fischer Scientific (Pittsburgh, PA). Distilled de-ionized water was obtained from a Milli-Q purification system Millipore (Billerica, MA). All aroma standards were purchased from Sigma Aldrich (Allentown, PA). The deuterated D_2-IBMP standard was prepared as described elsewhere (*15*). Solid phase extraction (SPE) cartridges packed with 200 mg LiChrolut EN sorbent (Merck, Darmstadt, Germany) were purchased from VWR International (West Chester, PA). Certified food-grade silicone Silastic® Laboratory Tubing (7.92 mm ID x 12.7 mm OD) was purchased from Dow Corning Corporation (Midland, MI) and cut into pieces weighing 0.66±0.04 g. All winemaking materials were purchased from Scott Laboratories (Petaluma, CA).

Absorption of MPs by silicone from model juice

Duplicate, 1 L bench-top trials were performed with 40 g silicone/L juice at two temperatures (16 °C and 25 °C). The model juice contained 80 g/L fructose,

80 g/L sucrose, and 7.5 g/L tartaric acid adjusted to pH 3.50 with NaOH, which was then spiked with IBMP and IPMP to yield a final concentration of 50 pg/mL of MP. An untreated control (no silicone) was also prepared. Samples (10 mL) were collected at 0, 3, 6, 12, 24, 48, and 96 h post silicone addition for MP analysis. No fermentation was performed on these trials. At each temperature, the partition coefficient (log K_{sil-aq}) for IBMP and IPMP between the silicone and juice phases was calculated based on the following equation:

$$\log K_{sil-aq} = \text{Log}\left(\frac{[\text{MP}]\text{final} - [\text{MP}]\text{initial}}{[\text{MP}]\text{final}} \times \frac{\text{mass of silicone phase}}{\text{mass of aqueous phase}}\right)$$

Winemaking

Over several years, four winemaking experiments were performed to evaluate the effectiveness of silicone in absorbing MPs: ***1)*** 2007 Cabernet Franc rosé wine with native detectable IBMP, ***2)*** 2008 Chardonnay white wine spiked with IPMP to simulate MALB taint, ***3)*** 2008 Cabernet Franc red wine (skin-contact fermentation) with native IBMP, and ***4)*** 2010 Riesling white wine with native detectable IPMP from MALB taint.

General winemaking protocol incorporating pre-fermentation silicone treatment

The general protocol for winemaking included steps of silicone addition and removal. In all trials, silicone was added immediately after juice or must preparation and removed prior to nutrient addition. Silicone pieces were added loosely to the white and rosé juice and wrapped in cheesecloth prior to addition to the red must. Once fermentation commenced, the loose silicone

pieces floated to the surface as a result of CO_2 production, which facilitated its removal. Yeasts prepared by the suppliers' recommended methods were inoculated after silicone addition while diammonium phosphate (DAP) (0.75 g/L) and Fermaid K (0.25 g/L) were added when the total soluble solids had been reduced by approximately 30%. Juices or musts were fermented to dryness (< 1 g/L residual sugar) verified by Clinitest. At the end of alcoholic fermentation, wines were racked off the lees, and SO_2 was added (90 mg/L for white/rosé wines and 80 mg/L for red wine). Wines were then cold-stabilized at 3 °C for 2 to 3 months prior to bottling. Malolactic fermentation, barrel aging and filtration were not performed. At bottling, the cold-stabilized wine was racked, and SO_2 was added in the form of potassium metabisulfite (40 mg/L or white/rosé wine and 35 mg/L for red wine). All wines were bottled in a 750 mL glass bottle, corked with natural cork, and stored horizontally at 18 °C. The detailed conditions of each trial including basic juice parameters are summarized in Table 2 and sections 2.3.2 to 2.3.5.

Table 2 – Summary of fermentation parameters used during silicone-fining studies

[1]Trial	Wine style	Juice parameters			Fermentation		[2]Time	Yeast Strains	[3]SO_2
		°Brix	TA(g/L)	pH	Temperature	Volume			
2007 CF	Rosé	23.1	[4]7.5	3.55	18 °C	15 & 45 L	18 h	Lalvin RC 212	Yes
2008 Ch	White	20.8	7.1	3.15	16 °C	1.8 L	13 h	EC1118	Yes
2008 CF	Red	22.7	7.9	3.34	9 - 29 °C	100 L	48 h	Lalvin RC 212	No
2010 R	White	21.4	8.7	3.08	16 °C	18 L	40 h	Epernay II	Yes

[1]CF: Cabernet Franc, Ch:Chardonnay, R:Riesling. [2]Time before yeast inoculation. [3]SO_2 (30 mg/L) added prior to silicone treatment. [4]3 g/L of tartaric acid added to must.

2007 Cabernet Franc rosé wine

Juice preparation: Machine harvested Cabernet Franc grapes (Finger Lakes, NY) were crushed (Vaslin-bucher Delta E-4, KLR Machines Inc., Sebastopol, CA), macerated for 2 h at 16 °C, and then pressed at $3x10^4$ Pa for 30 min (Willmes Anlagentechnik WPP-6000, Lampertheim, Germany) at Anthony Road Wine Co. in Penn Yan, NY. Following SO_2 addition (30 mg/L), the pressed juice was clarified by spontaneous settling for 38 h before racking and silicone addition. The initial IBMP concentration of the juice was 7.7 pg/mL. No IPMP was detectable in the original juice. *Yeast inoculation*: Lalvin RC 212 (0.25 g/L) was inoculated after 18 h of silicone contact. *Fermentation volume and temperature:* Duplicate fermentations were carried out in glass carboys (15 L and 45 L) at 18 °C. *Silicone dose and contact time:* Juice was treated with 0 g/L and 53 g/L for 41 h. *Sampling points*: Samples were collected for MP analysis at 0, 18, and 41 h after silicone contact.

2008 Chardonnay white wine

Juice preparation: Frozen Chardonnay grape juice was purchased from Kamil Juices (Ontario, Canada). The juice was not clarified prior to use. Juice was spiked with an IPMP standard to yield a final concentration of 60 pg/mL. Following SO_2 (30 mg/L) addition, silicone was added. The juice was kept at 25 °C for 13 h. *Yeast inoculation:* EC1118 (0.6 g/L) was inoculated after 13 h of silicone contact. *Fermentation volume and temperature:* Duplicate fermentations were carried out in 1.8 L at 16 °C. *Silicone dose and contact*

time: Juice was treated with 0 g/L and 32 g/L silicone for 48 h. *Sampling points:* Samples were taken at 0, 13, 24, and 48 h after silicone contact.

2008 Cabernet Franc red wine

Must preparation: Machine-harvested Cabernet Franc grapes from the Finger Lakes, NY region were destemmed and crushed (Vaslin-bucher Delta E-4, KLR Machines Inc., Sebastopol, CA) at Anthony Road Wine Co. (Penn Yan, NY). The initial IBMP concentration of the must was 3.2 pg/mL. IPMP was undetectable. No SO_2 was added prior to silicone treatment. Following crushing, the must was allowed to sit uninoculated at 3 °C for 48 h ("cold soak") in the presence of silicone. *Yeast inoculation:* Lalvin RC 212 (0.25 g/L) was inoculated 48 h after crushing. *Fermentation volume and temperature:* Duplicate 100 L musts were fermented in 125 L open top stainless steel vats. During fermentation, the cap was punched down manually twice daily. *Silicone dose and contact time:* One of four silicone doses (0 g/L, 4.4 g/L, 13.3 g/L or 40 g/L) was added to the must following crushing for 168 h. *Sampling points:* Must was sampled at 0, 2, 4, 5, 6, and 7 days after silicone contact. An additional sampling point was collected at day 11, the end of alcoholic fermentation. During fermentation, the temperature of the musts increased from 9 °C up to 29 °C on Day 7^{th} and then gradually decreased to 18 °C at the end of alcoholic fermentation. At the end of fermentation, the solids were removed by filtration through cheesecloth. The wine was then cold stabilized in glass carboys.

2010 Riesling white wine

Juice preparation: Machine harvested Riesling grapes (Finger Lakes, NY) were crushed (Vaslin-bucher Delta E-4, KLR Machines Inc., Sebastopol, CA), macerated for 2 h at 16 °C, and pressed at $3x10^4$ Pa for 30 min (Willmes Anlagentechnik wpp-6000, Lampertheim, Germany) at Anthony Road Wine Co. (Penn Yan, NY). The initial native IPMP concentration was 2.7 pg/mL as a result of visible multi colored Asian Ladybeetle contamination. No IBMP was detectable. Following SO_2 addition (30 mg/L), the pressed juice was clarified by spontaneous settling for 24 h at 16 °C. *Yeast inoculation:* Epernay II (0.25 g/L) was inoculated after 40 h of silicone contact. *Fermentation volume and temperature:* Juice (18 L) was fermented in glass carboys at 16 °C. *Silicone dose and contact time:* One of four silicone doses (0 g/L, 10 g/L, 20 g/L or 40 g/L) was added to the juice for 64 h. *Sampling points:* Juice was collected at 0, 16, 40, and 64 h after silicone contact.

Quantification of IBMP and IPMP

IBMP and IPMP in juices, musts and wines were quantified by head space solid phase microextraction (HS-SPME) coupled to GC×GC−TOF-MS using a D_2-IBMP internal standard, described in detail elsewhere (*15*). The analytical limit of detection was 1.2 pg/g for IBMP and 0.9 pg/g for IPMP. For aged wines, the final MP and SPE-GC-MS volatile analyses, described below, were performed in 2010.

Semi-quantitative analysis of other wine volatiles

Semi-quantitative analysis of other volatiles was carried out by a solid phase extraction (SPE) method described elsewhere (*39*). A 50 mL wine sample was spiked with 20 µL of internal standard mixture prior to SPE. The standard mixture was prepared in methanol containing 3-methyl-3-pentanol (200 mg/L), pentanoic acid (400 mg/L), 2-octanol (200 mg/L), 3-ethyl-3-dodecanol (200 mg/L) and 2-*sec*-butyl-phenol (200 mg/L) used to quantify alcohols, fatty acids, esters, monoterpenes, C_{13} norisoprenoids, vanillin derivatives and volatile phenols, respectively. SPE cartridges containing Lichrolut EN sorbent (200 mg packed in 3 mL cartridge) were conditioned with 5 mL dichloromethane, methanol and water consecutively prior to sample loading. SPE extractions were performed on a Varian 24-cartridge Positive Pressure Manifold (Palo Alto, CA). After loading, cartridges were dried under a N_2 stream for 20 min and then eluted with 1 mL dichloromethane. An aliquot of the extract (1 µL) was injected into a GC-TOF-MS (Pegasus, Leco, St. Joseph, MI). The column was a DB-Wax (60 m × 0.25 mm × 0.50 µm, Varian, Walnut Creek, CA), attached to a VF-17 ms (1 m × 0.1 mm × 0.2 µm, Varian). Although the system was set up for GCxGC analyses, the GC×GC modulator was turned off during analysis resulting in 1-D GC−TOF-MS. The sample was injected splitless with an injector temperature set to 250 °C. The purge was opened after 2 min. Helium was used as a carrier gas at a flow rate of 1 mL/min. The temperature program was as follows: initial hold for 1 min at 55 °C, followed by 3 °C/min to 240 °C, 30 min hold. The secondary column

and modulator temperature offset was +20 °C. The MS transfer line temperature was 260 °C. The TOF-MS was operated in EI mode with an ionization energy of 70 eV. The electron multiplier was set to 1700 V. MS data from *m*/*z* 20−400 were stored at an effective sampling rate of 5 Hz. Compound identification was performed by NIST library search in combination with literature retention index match and/or authentic standard verification. The unique ion, determined by the Leco Pegasus software, was used for peak integration.

Statistical Analysis

Statistical analysis was performed by JMP version 8 (SAS Institute, Cary, NC) using ANOVA and t-tests; comparison of means was analyzed by Tukey HSD.

Results & Discussion

Absorption of MPs from model juice by silicone

Duplicate bench-top experiments were conducted to evaluate the ability of silicone tubing to absorb IBMP and IPMP from a model juice at 16 °C and 25 °C over a period of 96 h. The resulting plots of MP vs. time for the treated and control juices are shown in Figure 1. The plots have the form of a decaying exponential, with faster extraction at the higher temperature, as is expected from a diffusion-limited absorption process. At both temperatures, 50% of each MP was removed by 8-12 h. As mentioned in the introduction, pieces of food-grade silicone tubing were used because they imparted no obvious off-aroma

and were relatively inexpensive. However, the tubing has relatively thick walls (4.7 mm) and low surface area, which should result in poor mass transfer properties. By comparison, debittering of orange juice using cellulose acetate particles of much smaller dimensions (0.075-0.210 mm) than silicone resulted in a 50% reduction of limonene within 10 min (*40*) or a factor of 48-72 times faster than the time necessary to reduce MPs by 50% in our study. For commercial applications, improved geometries of silicone (smaller particle size, greater surface area) should result in increased extraction rates. The treated wine in our study was not agitated, so it is also possible that extraction kinetics were limited by diffusion through the liquid phase.

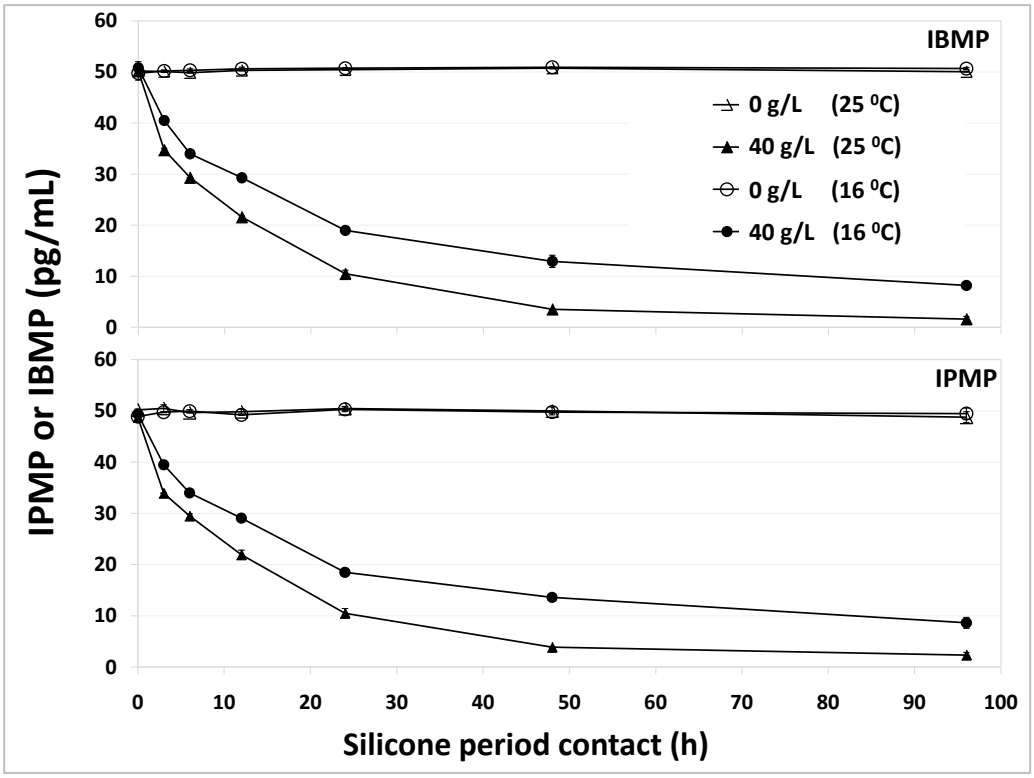

Figure 1 – Effect of silicone addition (40 g/L) on concentration of IBMP (top) and IPMP (bottom) in model juice over a contact period of 96 h at 16 ^{0}C and 25 ^{0}C. Standard deviations (n=2 trials) are too small, not visible.

In the 25 °C model juices, MP concentrations did not change significantly (one-tailed t-test, $p<0.05$) between 48 and 96 h, indicating that equilibrium had occurred between the solid and liquid phases. Less than 5% of the initial MPs were still present in the must after 48-96 h contact time. The MP concentration did not appear to have reached equilibrium in the 16 ºC juice by 96 h. The 96 h and 25 ºC data was thus used to calculate the silicone-juice partition coefficients for the MPs. Log K_{sil-aq} for IBMP was 2.21±0.4 and for IPMP was 2.05±0.6, similar to the literature log P value for IBMP, 2.44±0.09 (*20*). Our results are similar to a previous report which used synthetic corks to absorb MPs from wine and reported a 70-89% decrease in MPs after 160 h contact time following immersion of 5 or 10 synthetic corks in 1 L of wine (*28*). In our lab, we measured the mass of a synthetic closure as 6 g, which would equate to an addition rate of 30 or 60 g/L. From this, we can calculate that log K was ~2 in the previous work, comparable to our observed values. The key difference between the current study and this previous report is the timing of the polymer addition – in the current study, the polymer is added prior to fermentation, which avoids non-selective losses of fermentation derived volatiles.

Absorption of MPs from grape juice/must by silicone

Because our goal was to provide a 'proof-of-principle' demonstration that juice silicone fining could be used as a general strategy for selectively decreasing MP concentrations in wines, we used a wide range of fermentation parameters

such as fermentation volumes, temperatures, and skin contact times. Thus, we performed four trials over several years to evaluate the effectiveness of silicone in absorbing MPs from must and juice: 1) 2007 Cabernet Franc rosé wine (no skin contact) with native IBMP, 2) 2008 Chardonnay white wine spiked with IPMP to simulate MALB taint, 3) 2008 Cabernet Franc red wine (fermented on skin) with native IBMP, and 4) 2010 Riesling with detectable IPMP from MALB taint. Results of the trials, including the initial and final MP concentrations in the control and treated wines as well as fermentation details are summarized in Tables 2 and 3, respectively. A wide range of conditions was used in wine production to help gauge the appropriateness of silicone fining with a variety of fermentation techniques.

MP absorption from 2007 Cabernet Franc rosé

Following pressing and settling of juice, the 2007 Cabernet Franc rosé had an initial IBMP concentration of 7.7 pg/mL. No IPMP was detectable in the juice. A plot of IBMP vs. time for silicone treated (53 g/L) and control juice is shown in Figure 2A. We observed a 64% reduction (Anova, $p<0.05$) of IBMP after 18 h of silicone contact, at which point the must was inoculated. A reduction of >90% was observed (IBMP < 1 pg/mL detection limit) after 41 h, at which point the silicone was removed because fermentation had begun. The amount of IBMP absorbed at each time point was similar to values observed with model juice (Figure 2A). The untreated control, showed a small (5.2%) but significant

reduction in IBMP after 41 h, potentially from binding to yeast lees or volatilization.

MP absorption from 2008 Chardonnay white

In 2008, frozen Chardonnay juice was spiked with 60 pg/mL IPMP to simulate MALB taint. No IPMP was observed in the original juice. Again, significant reductions in IPMP were observed at the first sampling point (67% after 13 h) and the second sampling point (93% after 48 h), after which silicone was removed (Figure 2B). However, we noted a significant, though smaller, reduction in IPMP in the control sample, too (37% after 24 h and 50% after 48 h). At this point, fermentation had just begun – soluble solids had decreased by 1.5 °Brix – so loss due to entrainment in CO_2 seems unlikely. More likely, a portion of IPMP was lost via binding to the grape solids. In contrast to the clarified 2007 Cabernet Franc must, a noticeable amount of solids settled out from the juice prior to inoculation, although these solids were not characterized. A previous study reported a 38% decrease of IPMP after 24 h settling and a 50% decrease after 48 h settlement (*41*). Similarly, Roujou de Boubee, et al. had previously reported a 50% decrease in IBMP during cold settling of turbid Sauvignon Blanc juice (*16*).

MP absorption from 2008 Cabernet Franc red

Red wine production provides an additional challenge to MP removal, since MPs must first diffuse into the bulk liquid phase before they can be absorbed.

This limitation was previously observed in HS-SPME analyses of grape macerates (*15*). This behavior also appears to occur during red winemaking in the presence of silicone, demonstrated in the plot of IBMP vs. time for the different treatments (Figure 2C). Unlike the 2007 Cabernet rosé and 2008 Chardonnay wines, which were made without skin contact, the IBMP concentration of the 2008 Cabernet Franc red wine first increased as MPs were extracted from the skins during maceration. A previous study reported that IBMP reached a maximum after 24 h at 25 °C (*14*). In our current work, the IBMP concentration of the liquid phase slowly reached a maximum over five days, and the slower extraction kinetics are likely a result of the initial cooler temperatures in our study. Following crushing, the musts were at 3 °C, and were neither immediately inoculated nor heated. After 48 h, the must was inoculated, and the must gradually warmed to 29 °C by Day 7 (Figure 2C). Our rationale behind performing a “cold soak” was to allow time for the MPs to be extracted from the skins and absorbed by the silicone before fermentation commenced. However, this approach was not entirely successful, as we observed no significant differences among treatments for the first five days, based on a Tukey test (Figure 2C). A significant correlation of dose and IBMP concentration was observed at Day 4 and Day 5 based on a linear regression ($p<0.05$). Minimal alcoholic fermentation appeared to occur during this time. This lack of an effect occurred even though IBMP was initially observable in the free run juice (3.2 pg/mL), indicating that the cooler initial temperatures slowed the diffusion of IBMP from the must into the silicone.

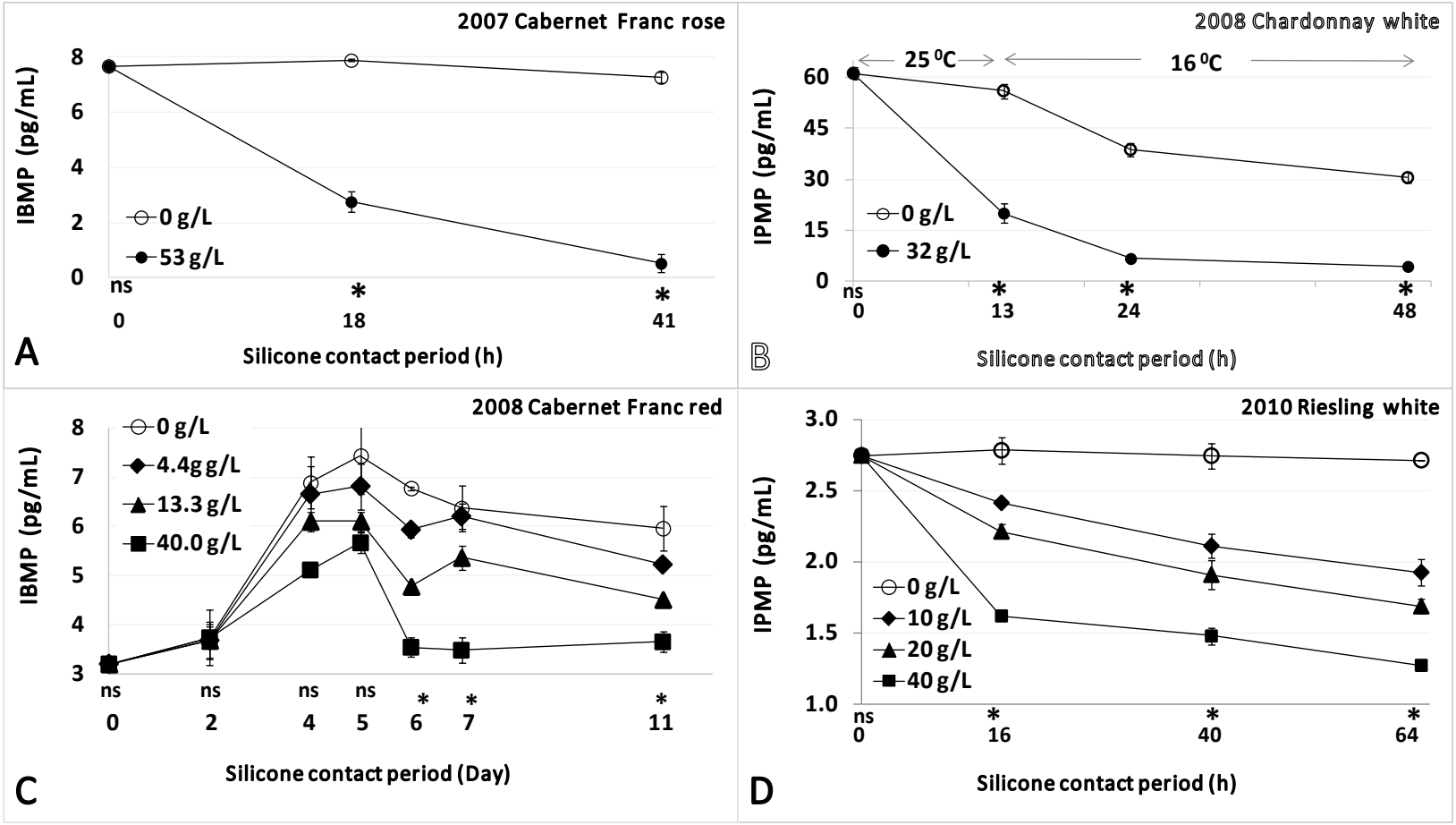

Figure 2 – Effects of pre-fermentation silicone treatment on **A)** IBMP in 2007 Cabernet Franc rosé without skin fermentation, **B)** IPMP in 2008 Chardonnay without skin fermentation, **C)** IBMP in 2008 Cabernet Franc red with skin fermentation, and **D**) IPMP in 2010 Riesling without skin fermentation. Silicone was removed after 41 h (**A**), 48 h (**B**), 7 d (**C**), and 64 h (**D**). * indicates significant differences among treatments at a time point ($p<0.05$), **ns** indicates no significant difference. Error bars represent standard deviations

Using a form of silicone with greater surface area should increase the rate of absorption and potentially make pre-fermentation cold soak with silicone a viable option. Even though no differences in IBMP were observed during the initial “cold soak”, IBMP could still be decreased significantly by silicone treatment well before fermentation completed. Alcoholic fermentation commenced between Day 4 (22 °Brix) and Day 6 (16 °Brix). Silicone was removed on Day 7, and thus would have avoided overlap with most of alcoholic fermentation. A significant decrease in IBMP was observed in both the 13.3 g/L and 40 g/L treatments as compared to the control from Day 6 until fermentation was complete. By the end of fermentation, the 40 g/L treatment had approximately half of the IBMP found in the untreated control (3.5 vs. 6.4 pg/mL).

MP absorption from 2010 Riesling white

In 2010, an infestation of MALB was noted in Riesling harvested from a Finger Lakes vineyard, and a characteristic “peanut/asparagus” taint was noted in the resulting juice. IPMP (2.7 pg/mL) was detected in the juice at concentration above its sensory threshold. A portion of this juice was used as a trial for pre-fermentation silicone fining on a real, contaminated commercial juice. Treatments included 10, 20, and 40 g/L silicone additions and an untreated control. However, due to technical problems, replicate fermentations were not available. As a result, statistical analysis relied on linear regression analysis. We observed a significant linear correlation between silicone addition rate and

IPMP decrease (p<0.001) at each time point (Figure 2D). The IPMP concentration in the 40 g/L treatment following fermentation was 1.2 pg/mL, a 56% decrease in IPMP compared to the starting juice (Table 3).

Effects of post-fermentation storage on MP concentrations

The MP concentrations in juice and must at the time of silicone removal and the final concentrations of MPs in wines were well correlated (r^2=0.99, p<0.05, concentrations below LOD omitted from analysis), even though MPs in the 2007 and 2008 samples were not measured until 2010 (Table 3). In five of the six wines with detectable concentrations of MPs, no significant change in MP concentration was observed relative to the juice or must sample following silicone removal, even though over 2 years had elapsed for some samples.

Table 3 – Summary of results from 4 silicone-fining studies performed over 3 seasons

		Silicone dose/contact		[2]MP (pg/mL)				[4] MP in aged wine
[1]Trial	Type/Nature of MPs	(g/L)	h	Start	End	[3] MP decrease		(pg/mL)
2007 CF	IBMP/Native	53	41	7.7±0.2	<LOD	>90%	*	<LOD
Untreated control sample				7.7±0.2	7.3±0.2	5%	ns	7.7±0.1
2008 Ch	IPMP/Spiked	32	48	61.2±1.4	4.5±0.3	93%	*	4.7±0.7
Untreated control sample				61.2±1.4	30.5±1.6	50%	*	38.4±1.4
2008 CF	IBMP/Native	40	168	7.4±0.8[5]	3.5±0.3	53%	*	3.7±0.4
Untreated control sample				7.4±0.8	6.4±0.5	14%	ns	6.6±0.1
2010 R	IPMP/Native	40	64	2.7±0.1	1.2±0.0	56%	*	<LOD
Untreated control sample				2.7±0.1	2.7±0.0	0%	ns	2.4±0.2

[1]CF: Cabernet Franc, Ch:Chardonnay, R:Riesling. [2] MP concentration at start and end of silicone treatment, unless otherwise specified. [3]Results from t-test, except for 2010 Riesling. *= significant, p<0.05, and n.s. = not significant. [4] Analyses were performed in 2010. [5]Maximum IBMP concentration in control at Day 5 skin contact fermentation trial. [6]Significant correlation observed for linear regression of silicone dose X IBMP concentration (p<0.05).

In the remaining sample (2008 Chardonnay white untreated control), a 25% increase was observed (30.5 to 38.4 pg/mL) for unknown reasons. The strong agreement between MPs before and after storage indicates that pre-fermentation fining with silicone or another non-polar sorbent should be appropriate for reducing MP concentrations in finished wines even after prolonged storage.

Effects of silicone treatment on fermentation kinetics

Potentially, silicone additions could absorb nutrients or co-factors, leading to a slower fermentation. We monitored soluble solids (°Brix) and sample temperature (°C) for all four trials and observed no significant difference in soluble solids at any time point (Anova, $p<0.05$). The soluble solids and temperatures for the four treatments were within 0.3 °Brix and 0.5 °C of the mean value at each time point (data not shown).

Effects of pre-fermentation silicone additions on other wine volatiles

Although silicone can absorb MPs from juice, this property is useful only if it avoids non-selective losses of key odorants from the final wine. Previous studies have demonstrated that a significant reduction in MPs in wine can be achieved with appropriate sorbents, e.g., treatment of wines with activated charcoal or synthetic closures (*18, 28*). However, the selectivity of these approaches for treating wine is dubious, since other non-polar compounds such as esters, monoterpenes and C_{13} norisoprenoids could also be removed.

For example, activated charcoal reduced the concentration of MPs by 34%, but no decrease in the vegetal aromas was observed. While the chemical composition of the finished wine was not characterized, it seems likely that other wine odorants were also removed. To evaluate the selectivity of pre-fermentation silicone treatment, we semi-quantitatively measured wine volatiles in the 2008 Cabernet Franc red wine and 2010 Riesling. Linear regressions were performed to determine if there was a significant correlation between silicone dose and volatile concentration. Excluding MPs, only 4 of the 55 volatiles in the 2008 Cabernet Franc red showed a significant correlation (Table 4), and only 17 of the 79 volatiles detected in the 2010 Riesling showed a significant correlation (Table 5). Of the 2010 Riesling volatiles, only 11 had a significant inverse correlation between the silicone addition rate and volatile concentration. Many of the unaffected volatiles are reported to exist in excess of their sensory thresholds in some wines, including esters (isoamyl acetate, ethyl butyrate); fusel alcohols (methionol, phenylethanol); fatty acids (isovaleric acid, butyric acid); C_{13} norisoprenoids (TDN, β-damascenone) and monoterpenes (α-terpineol) (*42*). These compounds are secondary aromas derived from fermentation, and can be formed *de novo* by yeast metabolism (esters, fatty acids, fusel alcohols) or released from semi-polar glycosides during fermentation or storage (monoterpenes, norisoprenoids) (*43*). Thus, pre-fermentation fining with non-polar sorbents appears to be a promising approach to selectively reduce MPs in finished wines.

Table 4 – Impact of pre-fermentation silicone treatment on 2008 Cabernet Franc wine volatiles

Significant correlation[1]		**No significant correlation[1]**
Aroma classes	**Difference[2]**	***p*>0.05**
Pyrazine		
IBMP	-53%	
Alcohols		
		1-Butanol, 1-Penten-3-ol, 1-Pentanol, 3-methylbut-3-en-1-ol, 3-methylpentan-1-ol (*E*)-3-Hexen-1-ol, (*Z*)-3-Hexen-1-ol, (*E*)-2-Hexen-1-ol, 1-Heptanol, Benzyl Alcohol
2-Nonanol	-43%	
Fatty acids		
Isovaleric acid	-5%	Isobutyric acid, Butyric acid, Isovaleric acid, Pentanoic acid
Hexanoic acid	-12%	Hexanoic acid, Octanoic Acid
Monoterpenes		
Hydroxycitronellol	-15%	α-Terpineol, 6,7-Dihydro-7-hydroxylinalool
Esters		Ethyl propanoate, Ethyl isobutyrate, n-Propyl acetate, Isobutyl acetate, Ethyl butyrate, Ethyl 2-methylbutyrate, Ethyl 3-methylbutyrate, Isoamyl acetate, Ethyl hexanoate, Ethyl pyruvate, Ethyl octanoate, Ethyl 2-hydroxyisovalerate Ethyl 3-hydroxybutyrate, Isoamyl lactate, Ethyl methylthiopropanoate, Ethyl 2-furoate, Ethyl 3-hydroxyhexanoate, Diethyl glutarate, Ethyl hexadecanoate
Ketones + Aldehydes		Furfural, Methyl 2-furyl ketone, 5-Methyl furfural
Lactones		Butyrolactone, Pantolactone, γ-Nonalactone
C_{13} norisoprenoids		TDN, *cis*-Actinidiol, *trans*-Actinidiol
Vanillin derivatives		Vanillin, Ethyl vanillate
Volatile phenols		Methyl salicylate, Guaiacol, *o*-Cresol, Phenol, 2-Methoxy-4-Vinylphenol, Syringol

[1]Linear regression of all treatments (0, 4.4, 13.3, and 40 g/L). A full list of analytes at each treatment is provided in Supplementary Table 1. [2]Difference of the 0 g/L versus 40 g/L treatments

Table 5 – Effects of pre-fermentation silicone treatment on 2010 Riesling wine volatiles

Significant correlation[1]		**No significant correlation**[1]
Aroma classes	**Difference**[2]	***p*>0.05**
Pyrazine		
IPMP	-56%	
Alcohols		
1-Butanol	-15%	2-methylpropan-1-ol, 1-Penten-3-ol,
4-Hepten-1-ol	-30%	3-methylbut-3-en-1-ol,4-methylpentan-1-ol, (*Z*)-2-Penten-1-ol, 3-methylpentan-1-ol, 1-Hexanol, (*Z*)-3-Hexen-1-ol, 3-ethoxypropan-1-ol, (*E*)-3-Hexen-1-ol, (*E*)-2-Hexen-1-ol, 1-Heptanol, 6-methylhept-5-en-2-ol, 1-Octanol, 2,3-Butanediol, Methionol, Benzyl alcohol, Phenylethyl alcohol
Esters		
Methyl isobutyrate	+57%	Ethyl propanoate, Ethyl isobutyrate, n-Propyl
Ethyl 3-methylbutyrate	-28%	acetate, sec-Butyl acetate, Isobutyl acetate, Methyl 2-
Ethyl hexanoate	+32%	methylbutyrate, Ethyl butyrate, Methyl hexanoate, (*Z*)-
Ethyl dodecanoate	-74%	3-Hexenyl acetate, (*E)*-3-Hexenyl acetate, Ethyl 2-hexenoate, Methyl octanoate, Ethyl 3-hydroxybutyrate, Methyl decanoate, Ethyl 3-hydroxyhexanoate, Ethyl decanoate, Ethyl 9-decenoate, Ethyl hexadecanoate
Fatty acids		
Isobutyric acid	-32%	Butyric acid, Isovaleric acid, 2-Methylbutyric acid,
(*E*)-2-Hexenoic acid	-24%	Dodecanoic acid
n-Decanoic acid	+33%	
Monoterpenes		
Terpinediol I	+19%	Terpinolene, *cis*-linaloloxide (furanoid), *trans*-
Geraniol	-90%	linaloloxide (furanoid), 8-Hydroxylinalool Nerol oxide,
Terpinediol II	+43%	Linalool, Hotrienol, α-Terpineol, *cis*-linaloloxide (pyranoid), *trans*-linaloloxide (pyranoid),6,7-Dihydro-7-hydroxylinalool, Hydroxycitronellol
C_{13} norisoprenoids		
cis-Actinidiol	-51%	TDN, β-Damascenone, *trans*-Actinidiol
Volatile phenols		
Guaiacol	-42%	Phenol
p-Cresol	-29%	
2-Methoxy-4-vinylphenol	+26%	
Syringol	-37%	
Ketones + Aldehydes		2-Octanone, Furfural, Benzaldehyde
Lactones		Butyrolactone, γ-Octalactone, γ-Nonalactone

[1]Linear regression of all treatments (0, 10, 20, and 40 g/L). A full list of analytes at each treatment is provided in Supplementary Table 2. [2]Difference of the 0 g/L versus 40 g/L treatments

The lack of effect is further illustrated in Figure 3, which shows the relative concentration of IBMP and other selected volatiles for all 2008 Cabernet Franc treatments. Silicone reduces IBMP in a dose dependent manner, but it has a non-significant or trivial effect on a diverse range of other secondary volatile compounds, including *cis*-3-hexenol, ethyl 3-methylbutyrate, ethyl hexanoate, hexanoic acid, actinidiol, and guaiacol. While most compounds were not significantly affected by an increased silicone dose, there were some exceptions. The largest change in a volatile in the 2008 Cabernet Franc was for 2-nonanol, which was 43% lower in the highest silicone rate compared to the control. While this compound has been previously reported in wine, its origins are not well established.

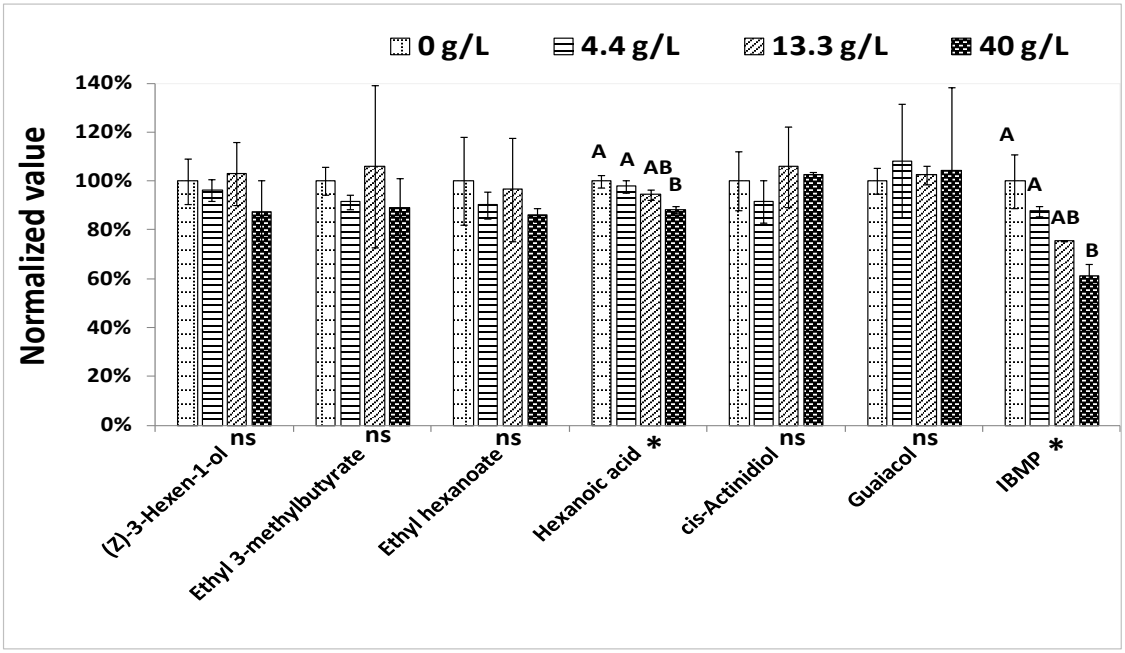

Figure 3 – Effects of pre-fermentation silicone treatment on selected volatiles in 2008 Cabernet Franc red wine (n=2). A full list of volatiles analyzed is in Supplementary Table 1. The Y-axis plots the ratio of the response for each volatile in each treatment against the mean response for the untreated control (0 g/L). The error bars reflect the standard deviation. * indicates significant difference (Anova & Tukey HSD) at $p<0.05$.

A likely source of this compound is via lipid degradation, which is well known for forming high concentrations of six carbon alcohols and aldehydes as well as lesser concentrations of nine-carbon compounds following crushing (*44*). During fermentation, aldehydes are further converted to corresponding alcohols. However, other lipid degradation products formed immediately after crushing, including *cis*-3-hexenol, were not affected by silicone in either wine. A potential explanation is that *cis*-3-hexenol or related compounds, are insufficiently non-polar to be extracted by silicone. One exception was a minor (12%) decrease in hexanoic acid, although this compound could be formed by yeast metabolism rather than lipid degradation. Interestingly, a significant correlation was observed between silicone dose and geraniol in the 2010 Riesling, with a 90% reduction observed at a 40 g/L dose, even though the other 12 monoterpenes measured were not significantly affected. Geraniol contributes to the muscat, floral aromas of some white aromatic varieties, including Riesling (*42*). A potential explanation is that the majority of geraniol in this Riesling sample exists in the free form rather than as a glycoside. Free geraniol has been previously detected in Riesling grape (*45*), although in muscat grapes, the bound form is reported to dominate (*46*). Potentially, bound geraniol was released less efficiently with our experimental winemaking conditions. Regardless, these results suggest that pre-fermentation silicone fining will likely be less appropriate on varietal wines which are dependent on primary aroma compounds.

Caveats for the use of silicone and other polymeric fining agents

Although food grade silicone is commercially available, its application as a processing aid or fining agent in winemaking processes is currently not approved by the United States Alcohol, Tobacco, Tax and Trade Bureau (TTB) or by regulatory agencies in other countries. Silicone is expected to non-selectively absorb non-polar compounds. Thus, while appropriate for treating juice and must, it is not suitable for selectively removing off-odorants from finished wines. As demonstrated in this paper, pre-fermentation silicone fining is more challenging with wines produced by skin contact fermentation. Because MPs must first diffuse from the skins before they can be absorbed (*15*), removal of MPs by silicone treatment may be improved by using winemaking techniques that more rapidly release MPs from grape skins. Under these conditions, quantitative extraction of MPs prior to fermentation may require increasing the extraction rate from the skins, i.e., by use of pectinases or increasing the temperature. Silicone treatment prior to fermentation may not be suitable for treating grapes where the varietal aromas of the finished wine are due to primary aroma compounds, such as monoterpenes in Muscat, rotundone in Shiraz, or methyl anthranilate in Concord (*38, 47*). Finally, the current silicone geometry needs to be improved to achieve higher surface area to increase extraction kinetics and decrease the silicone-juice contact time. Alternatively, other non-polar polymers like polyethylene should give similar results to silicone. These already exist in appropriate high surface area forms, but care should be taken, as we noted in

preliminary trials that high addition rates of polyethylene film led to noticeable plastic odor.

Conclusions

Treatment of grape juice and must with a non-polar sorbent – food-grade silicone tubing – prior to fermentation effectively absorbs MP without altering the concentrations of the majority of fermentation-derived compounds, and thus it appears to be a promising strategy for selectively reducing MP concentrations in finished wines. A number caveats exist to the use of silicone or other polymers. In particular, since other non-polar compounds in grapes could also be removed by this approach (e.g., methyl anthranilate, monoterpenes, and seqsuiterpenes) the approach should be most appropriate for wines that do not rely on primary grape aromas for their varietal character. Future work will investigate ways to improve the kinetics of MP extraction by developing appropriate food-grade, non-polar sorbents with greater surface area, as well as considering mass transfer of non-polar compounds like MPs from grape solids into the liquid phase. Finally, future work will also need to include sensory analyses of treated wines produced from grapes with both native and MALB derived MPs, to determine if the apparent selectivity of the approach translates into the desired sensory consequences of reduced herbaceousness.

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Chapter 3

Correlation of 3-Isobutyl-2-methoxypyrazine to 3-Isobutyl-2-hydroxypyrazine during Maturation of Bell Pepper (*Capsicum annuum*) and Wine grapes (*Vitis vinifera*)

Introduction

The 3-alkyl-2-methoxypyrazines (MPs), particularly 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (SBMP) are naturally occurring odorants with low orthonasal sensory thresholds (<10 pg/mL in water) and herbaceous aroma qualities (*1, 2*). The MPs are widely distributed in the plant kingdom, and can reach total concentrations in excess of 1000 pg/g in the vegetative tissue and unripe fruits of several plants, including bell peppers, lettuce, and asparagus (*3*). In recent years, the majority of reports on MPs have considered their role in wine grapes and wines, especially in the so-called Bordeaux cultivars (e.g. Cabernet Franc, Sauvignon blanc) (*4, 5*). In these varieties, the major MP species (IBMP) can be greater than 250 pg/g in unripe berries (*6*), and generally ranges from undetectable to 50 pg/g at harvest (*7*). The concentration of MPs in skin-fermented wines is highly correlated with concentrations in the original wine grapes (*8*). Since excessive MP concentrations will reduce consumer acceptance, and no satisfactorily selective method for removing MPs from wines has been established (*4*), there is interest in understanding factors that affect the

formation and disappearance of MPs in the vineyard (*6*). In wine grapes, MPs are reported to accumulate pre-veraison, and then to decrease markedly between veraison and maturity. Several environmental factors have been correlated with intermediate or final concentrations of MPs in grapes, including vine growth, temperature, and cluster light exposure (6-8). After reaching a maximum concentration pre-veraison, MPs are reported to decrease during ripening (*6*). Analogously, MP concentrations in red bell peppers are 4-fold lower than in green bell peppers (*3, 9, 10*). Whether this decrease is enzymatic or non-enzymatic is not yet established, as early studies suggested that MPs in grapes may be photodegraded *in vivo(11)*, but more recent reports have not supported this idea (*6, 12*). Mechanistic interpretations of these empirical observations have been handicapped by a poor understanding of MP biochemistry as neither the synthesis nor degradation pathways of MPs are clearly established in grape or in any other plant. Putative biosynthetic pathways for the MPs in plants were first proposed over 40 years ago (*3*). The initial steps are hypothesized to involve condensation of an alpha-dicarbonyl species with a branched chain amino acid (e.g. leucine) or its corresponding amino acid amide to eventually form a 3-alkyl-2-hydroxypyrazine (HP) and its 3-alkyl-2(1H)-pyrazinone tautomer (*13-16*). The HP is subsequently methylated to form the corresponding MP. While the initial cyclization step has not been confirmed in plants, an S-adenosyl-

methionine dependent methyltransferase capable of converting 3-isobutyl-2-hydroxypyrazine (IBHP) to IBMP has been isolated from Cabernet Sauvignon wine grapes (*17*). Recently, two cDNA ($VvOMT_1$ and $VvOMT_2$) encoding O-methyltransferases were reported to have a catalytic activity for MPs formation via *O*-methylation of its precursor, HPs (*18*). Regardless of the mechanism, the putative degradation product(s) of MPs have not been reported in the literature. In rats, IBMP is reportedly metabolized to IBHP and IBHP glycosides following ingestion (*19*). The demethylation of IBMP to IBHP effectively reverses the final putative step in IBMP synthesis. We hypothesized that a similar pathway in which MPs are degraded to their corresponding HPs may occur in plants during fruit ripening (Figure 1).

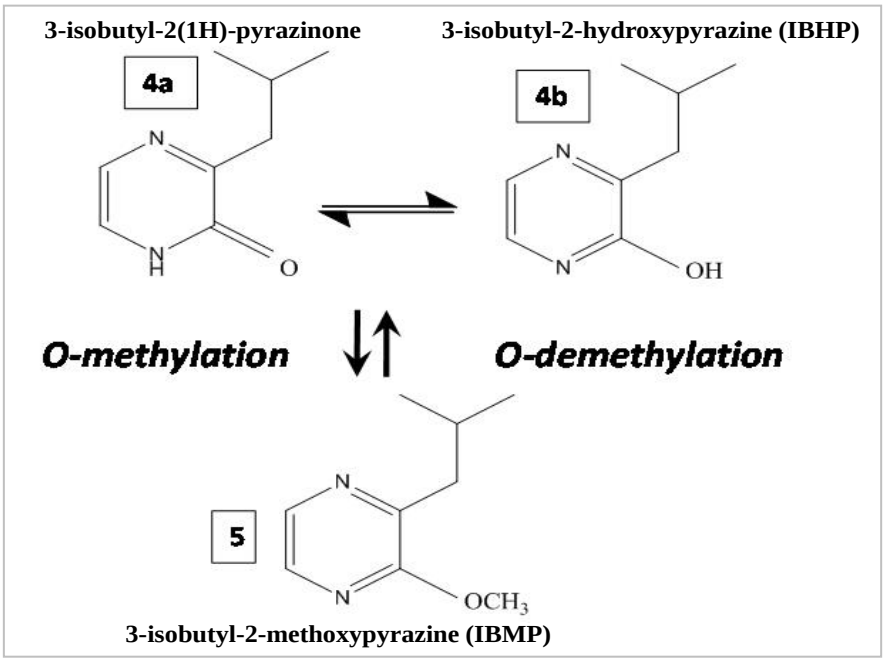

Figure 1 - Putative biosynthesis and degradation pathways of IBMP/IBHP through *O*-methylation or *O*-demethylation reactions. IBHP co-exists with its tautomer, 3-isobutyl-2(1H)-pyrazinone

If this pathway exists, then an inverse quantitative relationship should be

apparent between MPs and HPs during ripening. The presence of IBHP and 3-isopropyl-2-hydroxypyrazine (IPHP) has been previously reported in immature wine grapes (*13*), although not in bell peppers. The relationship of HP and MP during fruit maturation has not been studied. In this report, we demonstrate that IBMP to IBHP are inversely correlated during ripening in both wine grapes and in bell peppers, supporting the aforementioned hypothesis.

Materials and Methods

Chemical reagents and standards

Dichloromethane (DCM), methanol, pentane, ethyl acetate, citric acid, ascorbic acid and sodium hydroxide were purchased from Fischer Scientific(Pittsburgh, PA). DDI water was obtained from a Milli-Q purification system Millipore (Billerica, MA). Aroma standard, 2-*sec*-butylphenol was purchased from Sigma Aldrich (Allentown, PA). 3-isobutyl-2-hydroxypyrazine (IBHP) was purchased from Manchester Organics Ltd (97 %, Sutton Weaver, UK). 3-isopropyl-2-hydroxypyrazine (IPHP) and 3-*sec*-butyl-2-hydroxypyrazine (sBHP) were synthesized in our laboratory by condensation of either L-valinamide•HCl (97%, Sigma-Aldrich, St Louis, MO) or L-isoleucinamide•HCl (98%, TCI America, Portland, OR) respectively with glyoxal sodium bisulfate hydrate (Sigma-Aldrich) under alkaline conditions as described by Seifert et al. (*16*). The synthesized

products were confirmed by comparison of MS spectra to those in earlier reports and the purity of products was checked by GC-MS**.**

Fruit samples

Fresh bell peppers (*Capsicum annuum*) were purchased from a local market (Geneva, NY). For time-course studies on grapes, Cabernet Franc and Pinot noir were sampled from the Fox Run vineyard in the Seneca Lake AVA (Penn Yan, NY) and Riesling was sampled from Cornell University experimental vineyard in the Cayuga Lake AVA (Lansing, NY). Cabernet Franc was sampled pre-veraison (Aug 14th, 2009), post-veraison (Sept 23rd), and at harvest (Oct 13th). Pre- and post-veraison samples for Pinot noir were collected at the same date as Cabernet Franc samples. Riesling samples were collected at Aug 14th (pre- veraison) and Oct 20th (harvest). The sample size of Pinot noir and Riesling collected at harvest and pre-veraison respectively was insufficient for the IBHP analysis. Thus, these 2 data points were not available. Veraison was approximately Aug 18th for Pinot noir and approximately Aug 25th for Cabernet Franc and Riesling. The average growing degree days accumulated between bud-break and harvest at the two sites was 1202 GDD (base = 10°C). For recovery studies, frozen Cabernet Franc berries harvested in 2008 from Geneva, NY experimental vineyards were used.

Sample preparation of peppers and grapes

All fruit samples were kept frozen at -20 ^{0}C prior to sample preparation. For sample preparation, 100 g of pepper were blended in a Waring blender (Model No. 5011, Torrington, CT) in the presence of 50 mg/L ascorbic acid for 1 min and then filtered through cheesecloth. Filtered juice was loaded into 85 mL NALGENE polycarbonate centrifuge tubes (VWR International, West Chester, PA) and centrifuged for 30 min at 10,000 rpm and 5 ^{0}C (5810 R Centrifuge, VWR International). After centrifuging, the juice was filtered through a Whatman No. 41 filter paper. The supernatant was then subjected to solid phase extraction (SPE) or solid-phase microextraction (SPME) as described below. The sample size for grapes was larger than for bell pepper, but the protocol otherwise similar. Four kg of defrosted grapes were manually de-stemmed and blended in a Waring blender for 5 min in the presence of ascorbic acid (50 mg/L). The remaining steps for sample preparation were the same as that for bell pepper except 500-mL NALGENE centrifuge tubes were used. The speed of centrifugation on a larger rotor was adjusted to match the G-force of the smaller rotor and 85 mL centrifuge tubes.

Isolation of Free and Bound HPs from Juices

Due to the polarity of HPs, the SPE method of Ibarz et al. 2006 (*20*) for glycosides extraction was adopted for extraction of these compounds. All

SPE were performed on a Varian 24-cartridge Positive Pressure Manifold (Palo Alto, CA). SPE sorbent conditioning was performed by 2.5 mL dichloromethane, 2.5 mL of methanol, and 5 mL of H_2O per 100 mg of sorbent. Bell pepper juice (50 mL) was percolated through a 3-mL cartridge packed with 200 mg LiChrolut EN (Merck, Darmstadt, Germany). For grape samples, 1300 mg LiChrolut EN sorbent were manually packed into 12-mL cartridge, and 7 cartridges used for extraction of 1000 mL of grape juice. Extraction of both bell pepper and grape samples were carried out in duplicate. After sample loading, the sorbent bed was washed with 2.5 ml H_2O and 2.5 ml pentane:DCM (2:1 v/v) per 100 mg sorbent. Prior to elution, the bed was dried under pressure (25 psi, N_2) for 20 min. Subsequently, the targeted fraction was eluted with 4 mL (for bell pepper samples) or 25 mL (for grape samples) of ethyl acetate. For bell pepper samples, 20 µL of a 2-*sec*-butylphenol internal standard (50 mg/L in ethanol) was added to the 4 mL of eluent and concentrated to 0.3 mL with a continuous N_2 gas flow prior to GC-TOF-MS analysis. For grape samples, the 175 mL (25 mL x 7 cartridges) of eluent was concentrated to *ca*. 5 mL at 40 ^{0}C on a Buchi R-210 Rotavapor. Then, it was evaporated to dryness under N_2, reconstituted in 20 mL of 0.2M citric acid buffer adjusted to pH 2.5, and spiked with 20 µL of a 2-*sec*-butylphenol standard (50 mg/L in ethanol). The reconstitute was then subjected for second SPE (200 mg LiChrolut EN cartridge) preconditioned with 5 mL DCM, 5 mL methanol, and 5 mL H_2O. After

loading, the column was dried under N_2 (20 min, 25 psi) and HPs were eluted with 2.8 mL ethyl acetate. The eluent was concentrated to *ca*. 0.3 mL for GCxGC-TOF-MS analysis.

Analysis of Bound HPs from Juices

To determine if bound, acid-hydrolyzable HPs were present, an additional step was introduced following the initial SPE isolation step described in the previous section. Red pepper and Cabernet Franc juice samples were prepared separately from the previous study on free HPs. Following the initial SPE, the ethyl acetate fractions were evaporated to dryness and reconstituted with 10 mL and 20 mL citric acid buffer for the red pepper and Cabernet Franc samples, respectively. The buffer solution was heated in a 100 ^{0}C water bath for 1 h in an encapsulated vial under a N_2 -filled headspace, as described by Ibarz et al. (*20*). Twenty µL of 2-*sec*-butylphenol (50 mg/L) was then added to the hydrosylate prior to a 2nd SPE isolation on a 200 mg LiChrolut EN cartridge preconditioned according to the above procedure. The cartridge was dried under N_2 (25 psi, 20 min) and eluted 2.8 mL ethyl acetate which was subsequently concentrated under N_2 to *ca*. 0.3 mL prior to GC(xGC)-TOF-MS analysis. Additionally, we evaluated if free or acid-hydrolyzable bound forms of IBHP were not retained during loading or lost during the water wash prior to elution. To evaluate this, the water wash fraction was combined with the unretained

red pepper juice fraction, and a portion treated by the acid hydrolysis method described above. The hydrolyzed and non hydrolyzed juices were then re-extracted by the SPE method described in the previous method.

Quantification of HPs in SPE extracts by GC(xGC)-TOF-MS

Quantification of HPs in extracts was performed on a two dimensional gas chromatography coupled to a time-of-flight mass spectrometer, GCxGC-TOF-MS (Pegasus 4D, LECO Corp., St. Joseph, MI). For bell pepper analysis, HPs and MPs quantifications were performed in 1D with the modulator turned off. Grape samples were analyzed in 2D mode, GCxGC-TOF-MS. The 1st dimension column was a CP Wax 52CB (30m × 0.25 mm × 0.25 µm, Varian, Walnut Creek, CA) and the 2nd dimension column was a VF-17ms (2m x 0.1 mm x 0.2 µm, Varian). A programmable temperature vaporization (PTV) injector was used, with an initial injector temperature of 50 °C held for 0.5 min and then ramped at 200 °C /min to 250 °C. The injector was operated in pulsed splitless mode, where the injector pressure was held at 45 psi for 2.5 min. The purge was opened after 3 min. Helium was used as a carrier gas at a flow rate of 1 mL/min. The injection size was 1 µL. The temperature program was as follows: initial hold for 5 min at 55 °C, followed by a 10 °C/min ramp to 100 °C; then, 3 °C/min to 240 °C, 30 min hold. The MS transfer line temperature was 260 °C. The TOF-MS was operated in EI mode with ionization energy of 70 eV. The electron

multiplier was set to 1700 V. MS data from *m/z* = 20−400 was stored at 5 and 150 Hz for 1D and 2D analyses respectively. For GCxGC analysis, the modulation period was set for 3 seconds with a 0.75 s hot pulse time. The secondary oven temperature offset and modulation temperature offset were set for +20 ^{0}C. Data processing was carried out by the LECO ChromaTOF software. The qualifier ions were as follows: for IBHP *m/z* 110 (100), 137 (24), 81 (19), 152 (9), for IPHP, *m/z* 123 (100), 95 (71), 110 (52), 138(37), and sBHP were *m/z* 124 (100), 110 (65), 95 (61), 137(39). The quantifier ion for all HPs was *m/z* 110. The qualifier ions for the internal standard 2-*sec*-butylphenol were *m/z* 121 (100), 150 (25), 77 (18), 103(17) and the quantifier ion was *m/z* 150. The tolerance for the qualifier ions was ±20%.

Recovery experiments for model juice and green pepper juice

We evaluated recovery of IBHP by the SPE methodology in model juice (6 g/L tartaric acid, 50 g/L glucose and 50 g/L fructose, pH 3.5 adjusted with NaOH) and green pepper juice. Fifty mL of juice matrix was spiked with 20 µL IBHP stock solution in ethanol (25 mg/L) resulting in a spike of 10 ng/mL. IBHP was extracted and quantified by SPE followed by GC-TOF-MS as described in the previous sections. A “spiked reference” was also prepared, where IBHP was added to the ethyl acetate eluent prior to evaporation. Duplicates of both spiked juice and spiked reference samples were analyzed. The percent recovery was calculated as following:

$$Recovery\ of\ IBHP = \frac{Spiked\ Juice\ Matrix\ IBHP - native\ \ IBHP}{Spiked\ Reference\ IBHP - native\ IBHP} \times 100\%$$

Recovery experiments for grapes

Due to the large sample sizes (1000 mL) needed for IBHP analysis in grapes, it was more feasible to prepare calibration curves in citric acid buffer, skipping the first SPE extraction step. This necessitated determination of IBHP recovery to correct for losses during the first SPE step. Grape juice was prepared from 2 x 7 kg batches of fruit as described above. For each replicate, 1000 mL grape juice was spiked with 15 µL IBHP stock solution in ethanol (25 mg/L), resulting in a spike of 375 pg/mL. As described in previous sections, the juice was loaded onto a SPE cartridge, eluted with ethyl acetate. The solvent was evaporated, the extract reconstituted in citric acid buffer, then extracted on a 2nd SPE cartridge prior to analysis by GCxGC-TOF-MS. A "spiked reference" was also prepared, where IBHP was added to the citric acid buffer prior to the second SPE. The percent recovery was calculated as described for model juice.

Calibration curves and limits of detection for IBHP

The calibration curves were prepared in duplicate in either model juice (for bell pepper) or citric acid buffer spiked with grape extract for quantification of IBHP in grapes. The latter was prepared to mimic the matrix of grape samples. One kg of harvest-ripe *Vitis vinifera* L. cv. Pixie gapes was

processed into juice and extracted by SPE according to the sample preparation section. The SPE eluent was evaporated, reconstituted with 20 mL citric acid buffer, and heated to 100C for 1h to produce the grape extract spiked citric acid buffer. The calibration curve concentrations for grapes were duplicates of 0, 5, 25, 50, 100 and 200 ng/mL IBHP in citric acid buffer, equal to 0, 0.1, 0.5, 1, 2, and 4 ng/mL in 1000 mL grape juice assuming 100% recovery. The calibration curve for model juice was duplicates of 0, 5, 10, 20, to 60 ng/mL IBHP. Because the first SPE step for grapes was not employed when preparing calibration curves, the calibration curve was corrected for % recovery, calculated in the previous section. Limits of detection (LOD) were defined as the minimum peak area necessary to achieve a signal-to-noise ratio of 3:1, and were estimated from the calibration curves using Pallesen's method (*21*).

Extraction and quantification of IBHP in water-insoluble fraction of bell pepper

Red bell pepper was macerated as described in the previous section and filtered through cheesecloth. The filtered juice (120 mL) was centrifuged for 30 min at 10,000 rpm and 5 °C. The water-insoluble material retained on the cheesecloth was collected. Subsequently, the insolubles (13 g) were suspended in 26 g of 100% methanol and incubated at 25 °C for 2 hours with constant agitation at 200 rpm. The mixture was then centrifuged using

the same conditions as above. The supernatant was filtered and evaporated by Rotavapor at 40 °C for 15 min. One half of the juice fraction (60 mL) was used to reconstitute the extract. IBHP in the juice + insoluble extract was then analyzed by SPE followed by GC-TOF-MS and compared to IBHP in the other half of the juice sample with no added extract.

Quantification of IBMP in bell pepper and grapes

IBMP quantification in both bell pepper and grape samples were carried out by head space solid phase micro extraction (HS-SPME) coupled to GCxGC-TOF-MS using a deuterated internal standard, described in detail by Ryona et al. (*8*). The GCxGC modulator was turned off during analyses of bell peppers due to the higher concentration of IBMP and a lack of co-eluting compounds, resulting in 1-D GC-TOF-MS for these analyses. Additionally, an appropriate dilution with distilled water was used on bell pepper samples to keep IBMP within the calibration range (0 to 500 ng/L).

Reducing sugar quantification

The reducing sugar (fructose + glucose) content of bell pepper samples was measured enzymatically by a Glucose/Fructose UniFlex Reagent Kit (Unitech Scientific, Hawaiian Gardens, CA). For grape samples, an ATA-3810 PAL-1 Portable Digital Brix Refractometer (VWR international, West Chester, PA) was used to measure total soluble solids as a proxy for

reducing sugars.

Statistical analysis

Statistical analysis was performed by JMP version 8 (SAS institute, Cary, NC) using paired Student t-test, ANOVA and comparison of means by Tukey-Kramer HSD.

Results and Discussion

SPE Method Optimization

LiChrolut EN SPE has been previously used for recovery of polar compounds like furaneol and glycosides (*20, 22*), and these methods were adopted for isolation of HPs. The method utilizes H_2O for washing the cartridge prior to elution of the HP analytes. Since the HPs are moderately polar we were concerned they would be eluted prematurely during the wash step. To evaluate this hypothesis, the unretained fraction following percolation of red pepper juice through the SPE column was combined with the water wash fraction and treated with and without hydrolysis. The hydrolyzed and non hydrolyzed samples were then re-percolated through a new pre-conditioned SPE column. Results showed no detectable IBHP in this aqueous fraction for both hydrolyze and non hydrolyzed samples (data not shown). DCM, methanol and ethyl acetate were evaluated for their ability to elute IBHP from the SPE cartridge. Results showed a poor recovery of IBHP (<10%) using dichloromethane. Methanol showed a

modestly improved recovery of IBHP compared to ethyl acetate (~10%). However, the use of methanol required an additional step for removal of H_2O prior to GC analysis and also resulted in more peaks and higher signal-to-noise ratio in chromatography. Thus, ethyl acetate was chosen as the elution solvent in this study. To confirm that sufficient ethyl acetate was utilized during SPE, a 2.8 mL volume of ethyl acetate was used to re-elute a 200 mg LiChrolut EN SPE column that had been previously eluted with ethyl acetate. No IBHP was detected in the second elution. Although direct immersion solid-phase micro-extraction (SPME) was not attempted, the feasibility of headspace-SPME with both polar and non-polar fibers was evaluated at 40 ^{0}C for 30 minutes. No IBHP peak was visible using HS-SPME, likely because of the low volatility of IBHP. This limitation was not surprising and confirmed by the recent work where no presence of IBHP was reported in 16 *Capsicum* species utilizing the HS-SPME technique for extraction of volatile constituents (*23*).

Detection of HPs by GC(xGC)-TOF-MS in Juices

IBHP was readily detectable in green and red pepper juice using 1-D GC-TOF-MS, and its identity confirmed by comparison of retention time and mass spectra to an authentic standard. To our knowledge, this is the first confirmation of IBHP in a plant species other than *V. vinifera*. The concentration of IBHP was >2 orders of magnitude higher in our pepper

samples than in our grape samples. During sample preparation, a 20-fold greater volume of grape juice (1000 mL) than pepper juice was used to facilitate IBHP detection. However, a concurrent increase in interfering compounds was observed, such that no IBHP could be observed with 1-D GC-TOF-MS using standard polar and non-polar columns. Resolution of IBHP from other interferences could be achieved by 2-D GCxGC-TOF-MS using a strong polar X medium polar column set (CP Wax 52 CB X VF-17 ms). Figures 2A and 2B show representative contour and unfolded GCxGC chromatogram plots of IBHP, respectively, in post-veraison Cabernet Franc. Figure 2B depicts one of the modulated slices in the unfolded chromatogram, and clearly shows both quantifier ion and qualifier ions of IBHP (1^{st} RT 3526 s, 2^{nd} RT 2.076 s, and a 1^{st} dimension Kovats Index= 2713). Comparison of a citric acid buffer + Pixie grape extract with no detectable IBHP (Figure 2C) to the same sample spiked to 50 ng/mL IBHP (Figure 2D) confirmed the identification of IBHP in grape samples. GCxGC-TOF-MS permitted resolution of IBHP from a prominent interference (m/z 110, 64, and 81, 2^{nd} RT = 1.7 s), tentatively identified by NIST library search as catechol. Since this interference was not observed in bell pepper matrix, the catechol peak was thought to be derived from thermal degradation of anthocyanins or other polyphenolics. Other column sets were unsuccessful in resolving IBHP. The use of a non-polar VF-5 (2m x 0.1 mm ID x 0.4 µm) as the 2^{nd} dimension column with the

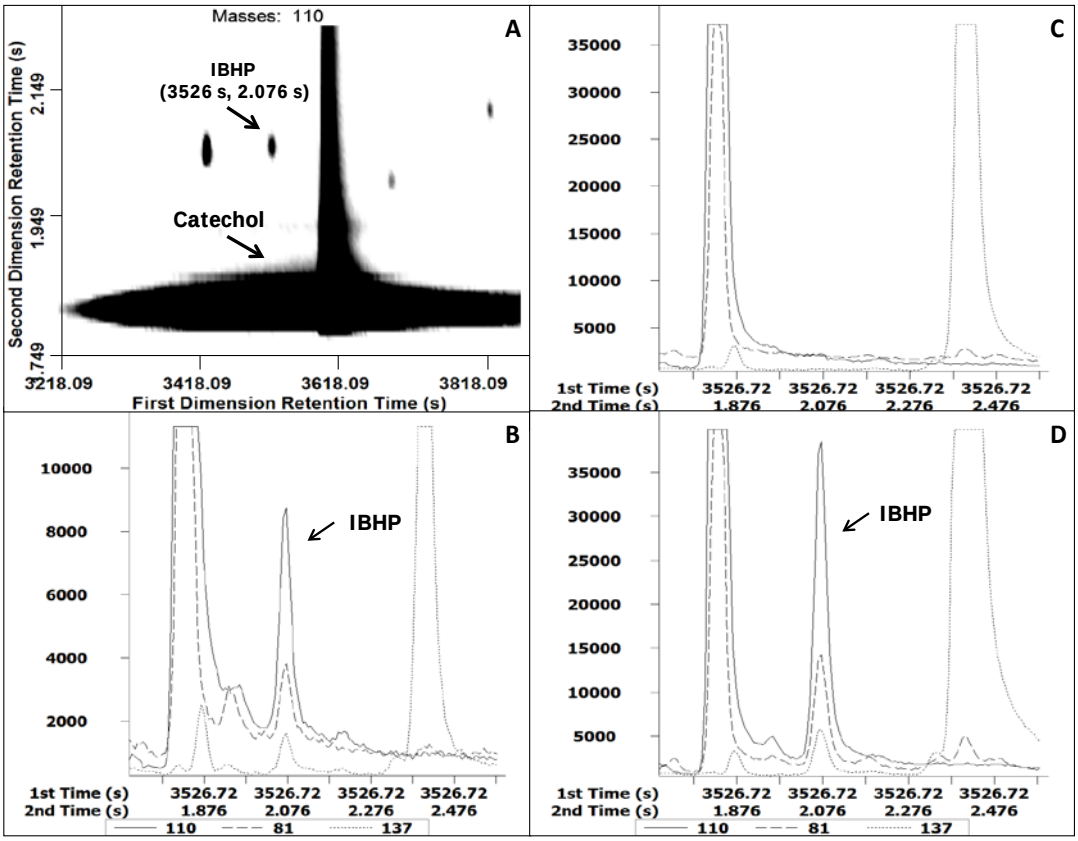

Figure 2 – (A) Contour plot displaying m/z 110 ion and (B) unfolded GCxGC chromatogram displaying m/z 110, 81, and 137 illustrating separation of IBHP from an interference (library identification as catechol) in post-veraison Cabernet Franc sample. Citric acid buffer with Pixie grape extract spiked with (C) 0 ng/mL IBHP and (D) 50 ng/mL of IBHP

CP Wax 52CB as the 1st column was not able to resolve this interference. A standard non-polar x polar column set, VF-5 X VF-17 ms, failed to separate IBHP from a different interference (data not shown). The other HPs, sBHP and IPHP, were not detectable in red pepper or grape juice samples, even when using GCxGC-TOF-MS for pepper samples. We had expected the ratios of different HP species to be proportional to the ratios of MP species. The ratios, IBMP:SBMP:IPMP, is reported to be approximately 100 : 1.5 : 1 in green peppers (*3*), 50: 0.15 : 1 in red peppers (*3*), and 36:5:1 in wine

grapes (*24*). With our current methodology, we may be below the detection thresholds for sBHP and IPHP. The detection thresholds were approximately 1 ng/mL in pepper (50 mL sample) based on thresholds for IBHP.

Recovery spikes, precision, and calibration curves in different matrices

Different recovery studies were performed to ensure that calibration curves prepared in model media appropriately reflected authentic juice samples. For pepper analyses, recovery experiments were performed using a model juice and green pepper juice, and recovery was calculated as described in the methods. Due to the tediousness of sample preparation for grapes, we prepared calibration curves starting with citric acid buffer, omitting the first SPE step. Recovery experiments were performed to determine IBHP loss during this initial step. Recovery of IBHP from the grape juice was 83.2 ± 11% (standard error), comparable to recovery of IBHP from the model juice, 79.7 ± 4.7% and recovery in green pepper juice, 80.8 ± 4.5%.

In model juice, calibration standards from 0 - 60 ng/mL IBHP resulted in a linear calibration curve (R^2= 0.998). The IBHP detection threshold for the pepper extraction protocol was determined to be 1 ng/mL. For grapes, calibration standards were prepared from 0 - 200 ng/mL IBHP in the citric acid buffer, or effectively 0 to 4000 pg/mL in grapes. Good linearity was achieved (R^2= 0.999) and the IBHP detection threshold in grapes was

calculated to be 25 pg/mL by Pallesen's method following correction for recovery. This relatively high LOD values of IBHP were partly due to co-eluted peak in grape sample and partly contributed by the poor mass spectral response factor which measuring the height of an analyte with respect to its concentration. The IBMP peak (*m/z* 124) showed 6 times higher response factor compared to IBHP peak (*m/z* 110) under 1 µL liquid injection at the same concentration.

IBMP versus IBHP concentration in bell pepper

As tissue disruption by freezing had been reported to change the volatile profile of C6 aldehydes and alcohol, IBMP concentration showed no difference between before and after freezing (*25*). In this study, analysis of IBMP and IBHP was conducted in *defrosted* bell pepper. Visual appearance was used to assay 5 different ripening stages (Figure 3), as reducing sugars did not prove to be a useful metric for ripening. Reducing sugars, glucose + fructose, ranged from 29.6 to 37.7 g/L in peppers, with a mean value of 34.8 g/L. The reducing sugar concentrations are 2 fold lower than those previously reported (*26*), and were not correlated with visual maturity, IBHP concentration, or IBMP concentration. During ripening, IBMP decreased from 86.6 ng/mL (green, Stage 1) to 15.4 ng/mL (red, Stage 5) with intermediate concentrations observed in Stages 2-4. The decrease in IBMP during bell pepper ripening has been previously reported (*27*).

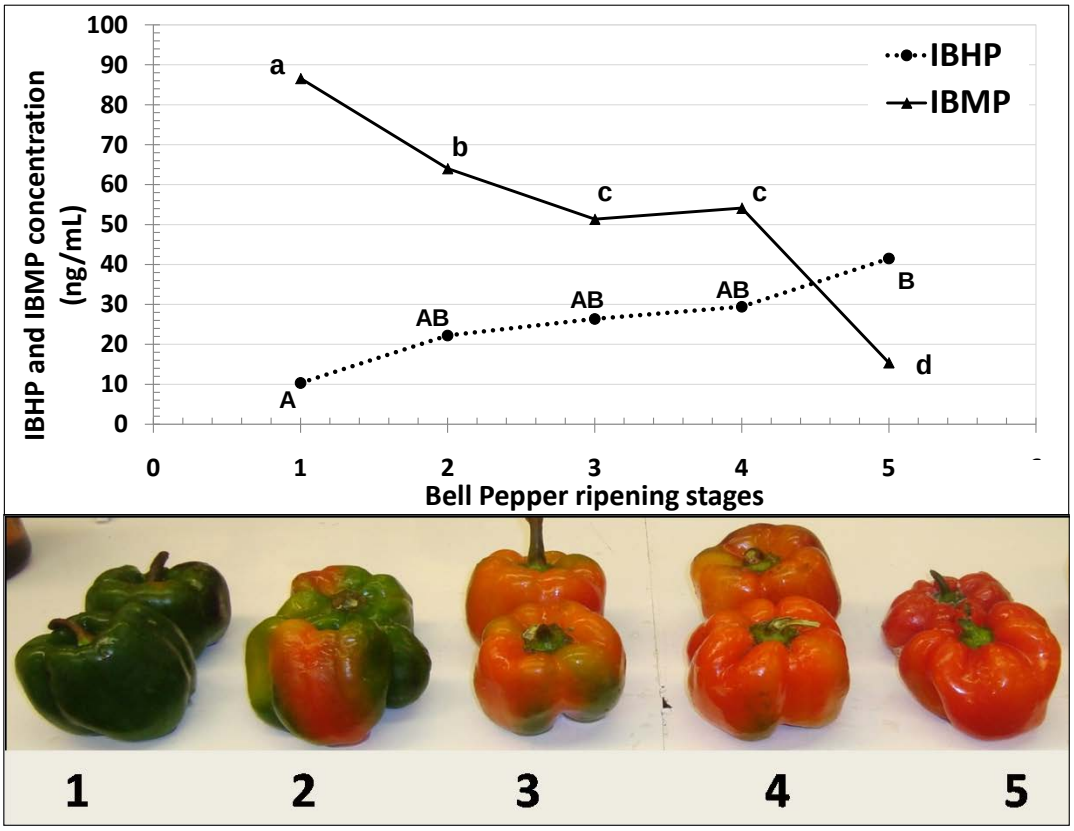

Figure 3 –IBMP and IBHP concentrations, ng/mL, in bell pepper during ripening at 5 maturity stages. Different letters indicate a statistically significant difference at $p<0.05$

The IBMP concentrations in peppers measured in this study were higher than previously reported concentrations, 20 ng/g and 5.5 ng/g in green and red peppers, respectively (*3*). This discrepancy could be due to biological variation, e.g. different cultivar, or it may be due to differences in sample preparation, as the earlier report used purge-and-trap for extraction and may not have quantitatively recovered IBMP. During pepper ripening (Figure 3), there was a corresponding increase in IBHP from 10.3 ng/mL (green, Stage 1) to 41.5 ng/mL (red, Stage 5). Stage 1 had significantly lower IBHP than Stage 5. Stages 2, 3 and 4, with a mixture of green and red color, demonstrated no significant difference in IBHP as compared to

Stage 1 or Stage 5. In addition to the five maturity stages, we also measured IBMP and IBHP in four bell peppers purchased from another supermarket: orange, yellow, another green, and another red bell pepper. A plot of IBHP vs. IBMP in the nine peppers (five maturity stages + four additional) is shown in Figure 4. Data points labeled with numbers refer to the bell pepper samples in Figure 3, while the other peppers are labeled with their color. We observe a significant inverse correlation (R^2 = 0.958) between IBMP and IBHP concentrations. The orange and yellow bell peppers had IBHP and IBMP intermediate between the green and red peppers (Figure 4). The highest IBMP (125 ng/mL) for all samples was observed in a green pepper sample.

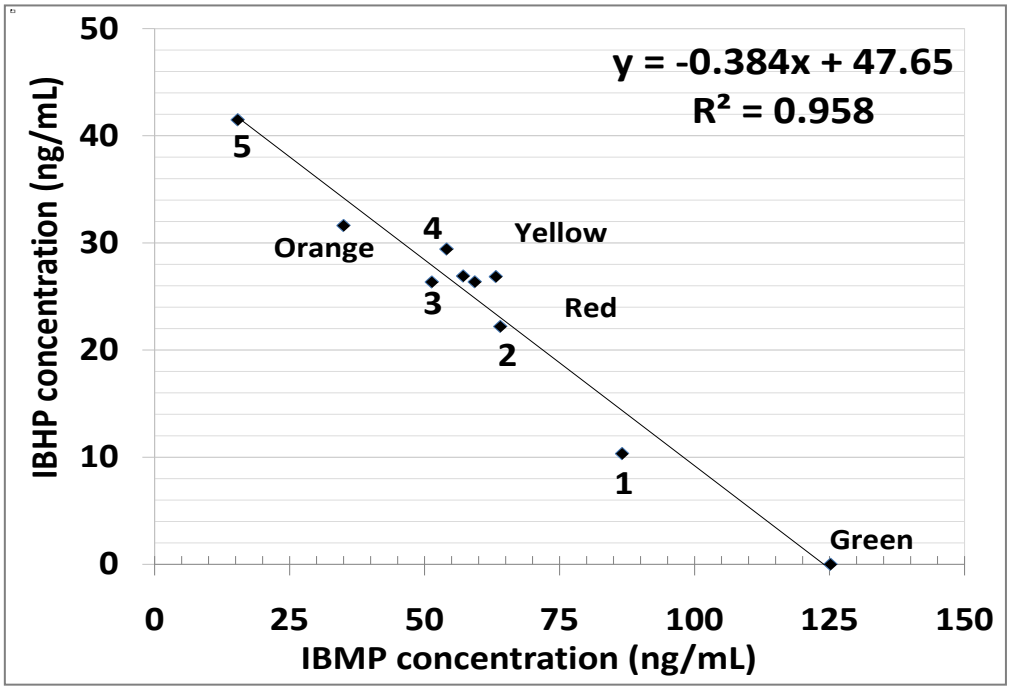

Figure 4 –IBMP versus IBHP concentrations in bell pepper sampled from different sources and at different maturities. Numbered samples correspond to their maturity stage in Figure 2. Samples labeled with a color name were not part of the Figure 2 maturity study, and the label refers to the color of the pepper

The same green pepper sample also had the lowest IBHP of any sample, beneath limits of detection. Conversely, the highest IBHP was observed in a red pepper, 41.5 ng/mL, and the same sample had the lowest IBMP, 15.4 ng/mL. The strong inverse correlation between IBMP and IBHP suggests that IBMP could be degraded to IBHP during ripening, a hypothesis which is discussed in more detail below.

Distribution of IBHP in Bell Pepper

When the insoluble portion of red bell pepper was extracted with methanol and dried, and the extract recombined with the original juice, we observed no difference in measured IBHP (data not shown). In grapes, IBMP is located primarily in berry skins, but is readily extracted into the aqueous juice fraction during maceration even prior to alcohol production (*28*). Since IBHP is more polar than IBMP, it is not surprising that IBHP partitioned quantitatively into the juice fraction.

IBMP and IBHP in *Vitis vinifera* grapes

Cabernet Franc, Pinot noir, and Riesling were collected at various maturity stages and analyzed for IBMP and IBHP (Table 1). The pre-veraison concentrations of IBMP were: Cabernet Franc (259 pg/mL), Riesling (71 pg/mL) and Pinot noir (11 pg/mL). During maturation, IBMP decreased to 30 pg/mL and then to 2 pg/mL in Cabernet Franc, and to below the limit of

detection (<1.2 pg/mL) (*8*) in Riesling and Pinot noir. The order, Cabernet > Riesling > Pinot, is in concordance with previous reports (*24, 29*). Similar to bell pepper, we observed a significant increase in IBHP during maturation of grapes. IBHP concentration at the last sampling point for each cultivar was significantly different among all cultivars. The same order for harvest IBHP was observed for pre-veraison IBMP, with the highest IBHP observed in Cabernet Franc (235 pg/mL), followed by Riesling (78 pg/mL), and undetectable concentrations in Pinot noir. Interestingly, we observed comparable concentrations of IBHP in all cultivars pre-veraison (64-88 pg/mL), with the highest concentrations in the non-MP accumulating Pinot noir. The final step of IBMP synthesis is proposed to be *O*-methylation of IBHP to form IBMP, and a previous study demonstrated a positive correlation (R^2= 0.779) of IBMP versus IBHP in 8 cultivars collected at 40 days post-bloom (*13*). However, the authors also reported that pre-veraison Pinot noir, Riesling, and Cabernet Sauvignon had comparable concentrations of IBHP (*ca.* 3 nmol/kg fresh weight, or ca. 500 pg/mL), despite differing by over an order of magnitude in IBMP. Therefore, the poor correlation we observed between pre-veraison IBHP and IBMP for the three cultivars selected in our study was not unexpected. However, the concentrations of IBHP in our study were a factor of 10 lower than those previously reported by Hashizume, et al. Similarly, up to 200 pg/L IPHP were detected in pre-veraison Riesling and Pinot noir in the

previous study, but no IPHP was detected in our current study. The reason for the quantitative discrepancy between these studies is not clear.

Presence of Acid-Hydrolyzable "Bound" IBHP in Grapes and Peppers

Previous work on the metabolism of IBMP in rats demonstrated that the IBMP was demethylated to IBHP and then partially glycosylated (*19*). Because other aromatic alcohols are reported to be glycosylated post-veraison in grape berries, e.g. guaiacol and 4-methylguaiacol associated with "smoke taint"(*30*), we hypothesized that IBHP may exist in a glycosylated forms in fruit. Following SPE, the hydrolysate (released of aglycone) through hydrolysis was then extracted via second SPE. This step of second SPE was needed for consistency and accuracy in comparison of hydrolyzed and non-hydrolyzed samples. To better understand whether the current method measuring free and/or bound IBHP, a study comparing hydrolyzed and non hydrolyzed samples was conducted on duplicates of 50-mL red bell pepper and 1000-mL post-veraison Cabernet Franc grapes. We observed a significant increase (p<0.05, one-tailed t-test) in the grape samples resulting from hydrolysis (143±10 pg/mL hydrolysis vs. 95±15 pg/mL non-hydrolysis) and no significant difference in red pepper (58±4 ng/mL hydrolysis vs. 53±8 ng/mL non-hydrolysis) (Figure 5).

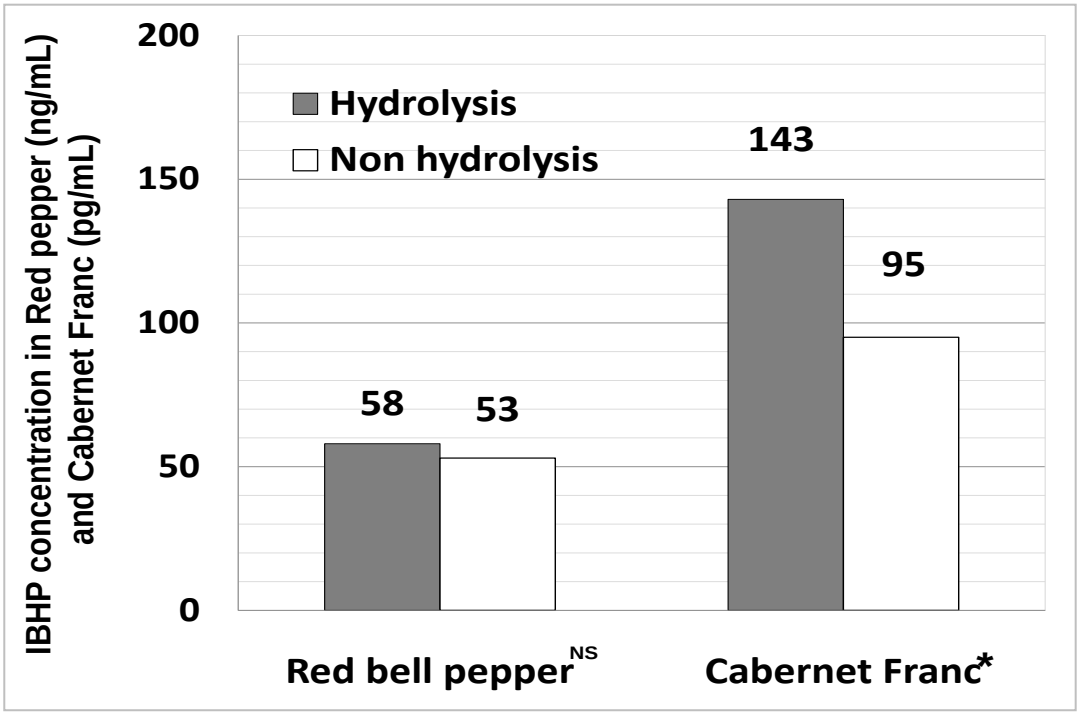

Figure 5 – Hydrolyzed and non-hydrolyzed IBHP levels in red bell pepper and mature Cabernet Franc. NS indicates no statistically significant difference and * indicates a statistically significant difference (one-tailed t-test, $p<0.05$). The error bar reflects standard deviation of the duplicates

Thus, a fraction of IBHP in grapes (33%) appears to exist in an acid-hydrolyzable bound form, potentially a glycoside. This distribution is similar to the excretion patterns of IBHP and its glycoside following IBMP metabolism observed in rats. Based on these results, we were concerned that the IBHP peak observed for grape samples (Table 1) may be partially derived from thermal degradation of IBHP precursor forms. However, increasing the cool hold time of PTV injector following injection resulted in a 2-fold decrease in peak area for both the IBHP peak and the *sec*-butylphenol internal standard, resulting in no change in IBHP quantification. By comparison, the catechol interference decreased markedly under the

lower injector temperature conditions, supporting the idea that the catechol peak is due to thermal degradation of polyphenols.

Evaluation of Hypothesis that IBMP is Demethylated to IBHP during Fruit Maturation

We undertook this study with the hypothesis that MPs are degraded to HPs and/or HP glycosides during fruit maturation. In rats, IBMP is reportedly metabolized to IBHP and IBHP glycosides following ingestion (*19*). If a similar phenomenon occurred in fruit, we would expect to see a quantitative increase in IBHP as IBMP concentrations decrease, which we observe. A plot of IBHP vs IBMP for Cabernet Franc yielded a strong inverse correlation, R^2 =0.998 (Figure 6A). Assuming that IBHP was not further transformed, we would also expect to observe a strong correlation between pre-veraison IBMP and final IBHP. We observe a significant, positive correlation, R^2=0.990, between pre-veraison IBMP and final IBHP across the 3 cultivars we studied with the order Cabernet Franc > Riesling > Pinot noir for both analytes (Figure 6B). Although further studies are clearly necessary, these data are compatible with the MP-to-HP degradation hypothesis. The data also indicate that IBHP concentrations at harvest could be used as a proxy for the maximum IBMP achieved pre-veraison. Our hypothesis would also predict that the total IBHP + IBMP (moles per ber ry) in fruit should not change during ripening.

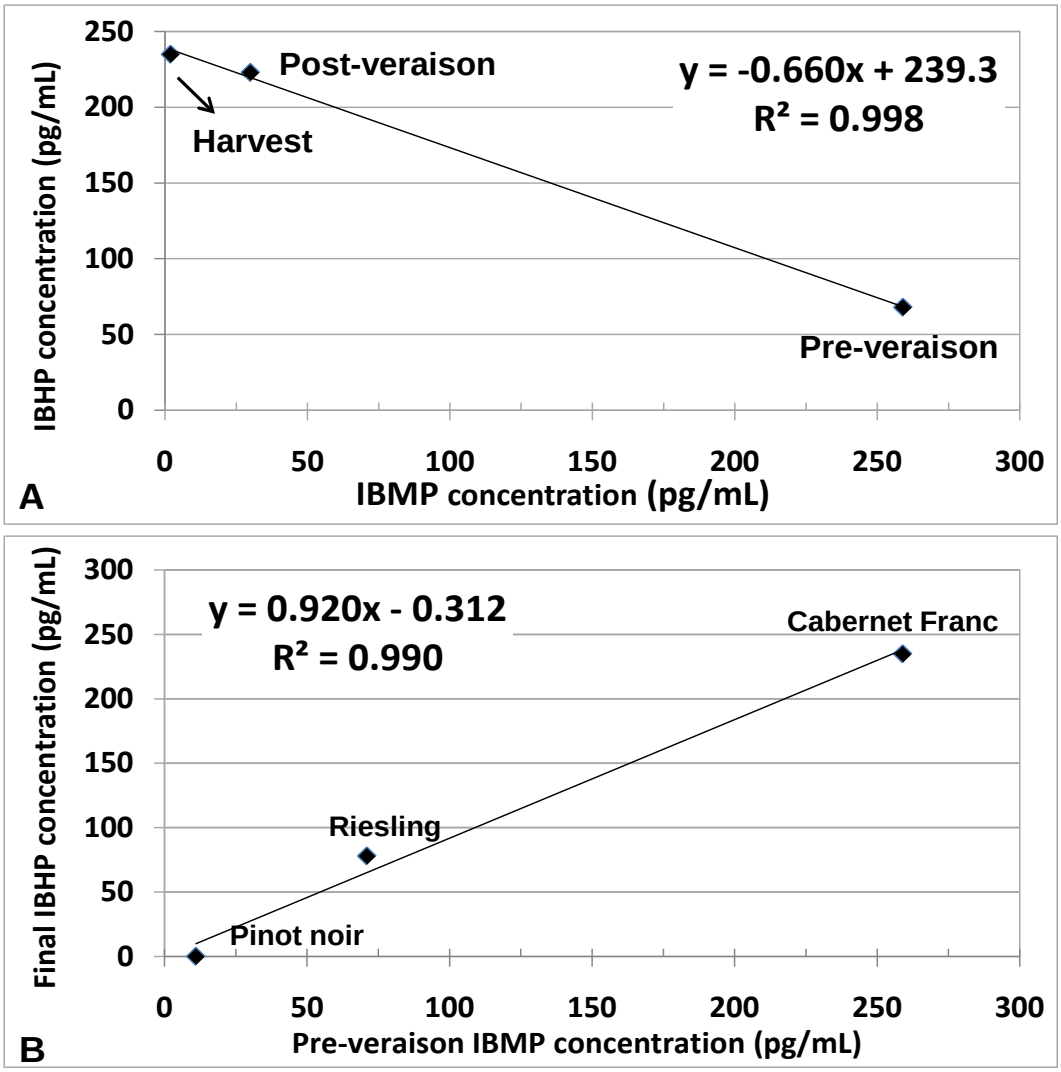

Figure 6 – (**A**) IBMP versus IBHP concentrations in pre- and post-veraison and harvest Cabernet Franc samples and (**B**) pre-veraison IBMP versus final IBHP concentrations in Cabernet Franc, Pinot noir and Riesling.

Unfortunately, we did not measure berry weights and thus cannot account for any dilution caused by berry enlargement, nor did we measure bound IBHP in the samples used for the maturity study data shown in Table 1.

The bell pepper data did not support the IBMP degradation hypothesis as clearly. While a very strong correlation was observed between IBMP and IBHP during maturation (Figure 4), the slope of the best-fit line was 0.38, and no bound, acid-hydrolyzable IBHP was detectable.

Table 1 – IBMP, IBHP, and total soluble solid concentrations in V*itis vinifera* grapes during berry maturation

	IBHP (pg/mL)	IBMP (pg/mL)	Total soluble solid (^{0} Brix)
Pre-veraison			
Cabernet Franc (Aug 14th)	68±1	259±6	5.3
Pinot Noir(Aug 14th)	88±10	11±0	5.5
Riesling (Aug 14th)	64±4	71±7	5.4
Post-veraison			
Cabernet Franc (Sept 23rd)	223±10	30±1	18.6
Pinot Noir (Sept 23rd)	<LOD	<LOD	18.4
Riesling	NA	NA	NA
Harvest			
Cabernet Franc (Oct 13th)	235±7	2±0	20.6
Pinot Noir	NA	NA	NA
Riesling (Oct 20th)	78±9	<LOD	20.7

<LOD means below limits of detection (25 pg/mL for IBHP, 1.2 pg/mL for IBMP). NA means samples not available for this study. All values of IBHP were quantified as free IBHP with no treatment of hydrolysis.

Therefore, if degradation of IBMP to IBHP does occur during pepper ripening, *ca*. 60% of the IBMP present in the green peppers cannot be accounted for in the ripened peppers (Figure 3). Potentially, the IBHP is further metabolized in peppers, or additional IBMP degradation pathways exist. A final possibility is that O-methyltransferase activity and consequent methylation of IBHP to IBMP decreases during ripening in both peppers and grapes; the increase in IBHP thus reflects a decrease in metabolic flux, resulting in a build-up of the IBHP intermediate. Labeled precursor studies, as well as more detailed time course studies, will be useful in refining these hypotheses.

Conclusions

In summary, we have demonstrated that IBHP concentrations increase proportionally to the decrease in IBMP in both ripening peppers and wine grapes. Although these results provide some evidence that MPs are possibly degraded to HPs during fruit ripening, future studies examining the enzymatic *O*-demethylation activity, which has been reported in plant metabolism (*31*), is warranted to validate this finding. Since OMT activity was reported to decrease sharply after 4 weeks post flowering in grapes, it is very unlikely that this enzyme is available for and/or capable of performing the reverse reaction (*18*). If our hypothesis is true, free IBHP at harvest could be used a proxy for the pre-veraison maximum IBMP. This would be useful for viticultural studies interested in determining factors that affect IBMP accumulation pre-veraison, as it would decrease the number of sampling time points necessary. Assuming that IBHP persists through during fermentation, it may even allow for *post hoc* evaluation of maximum IBMP concentrations in the vineyard by measuring IBHP in finished wines. In the case of Cabernet Franc wine grapes, we can detect a significant increase in IBHP following acid hydrolysis, indicating that IBHP may also partially exist as a glycoside, although this tentative conclusion should be confirmed, i.e. by synthesis of a standard and identification by LC-MS (*7*). Further studies on HPs in grapes will be facilitated by improved analytical methodologies. The current protocol requires 1 L of juice and 2-D GCxGC

to avoid co-eluting interferences. The use of a more selective clean-up protocol and/or use of LC-MS/MS could be appropriate for reducing sample size and avoiding interferences.

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Chapter 4

Glycosylated Aroma Compounds in Grape Juice and Wine: The Analytical Techniques and The Viticultural and Enological Impacts

Introduction

Composition of volatiles in wine grapes and wines is known to consist of both free and bound aromas (*1-5*). Free aromas are also known as the primary aromas as there are readily to be detected in grape juice prior to fermentation. On the contrary, bound aromas are neither volatile nor odorous. They consist of volatile constituents bound to the non-volatile counterparts such as a mono-saccharide and disaccharides. Examples of monosaccharide and disaccharides are β-D-glucopyranoside and α-L-arabinofuranosyl-β-D-glucoside, α-L-arabinopyranosyl-β-D-glucoside, β-D-apiofuranosyl-β-D-glucoside, or β-D-xylopyranosyl-β-D-glucoside, α-L-rhamnopyranosyl-β-D-glucoside (rutinoside) (*3, 6-8*). These mono- or di-saccharides are covalently bonded to the volatile molecules of various aroma classes such as monoterpenes, norisoprenoids, volatile phenols, and vanillin derivatives (*5, 9*). During the winemaking stages, bound aroma compounds are released via yeast metabolism and enzymatic or acid hydrolysis (*10-12*). During barrel and bottle aging, the process of acid hydrolysis will continue to release the remaining bound fractions (*13*). The bound volatiles in grapes are more abundant than the free ones present 2 to 8 fold higher concentration (*14, 15*). In many varieties, bound volatiles are responsible for the key aromas in wines

such as the fresh fruity and floral aromas in *Vitis vinifera* Riesling. There are exception for a few cultivars such as *Vitis vinifera* Cabernet sauvignon, gewürztraminer and *Vitis labrusca* Concord, where their respective varietal aromas of green peppery, fruity (lychee), and foxy are present in and are detected in the juice. The glycosylated bound volatiles are often referred as glycosides where the released fractions of the free counterparts are referred as aglycones. During grape ripening, most desirable aroma molecules start accumulating from veraison toward harvest while undesirable ones start decreasing. A clear example of this phenomenon is the accumulation of many monoterpenes and C_{13} norisoprenoids exhibiting fruity and floral aromas (*16, 17*) and the degradagation of vegetative aroma of methoxypyrazines (*18*). Both desirable and undesirable free and bound aroma molecules are mainly located in the skin of the berries (*14*). During juice processing steps such as crushing and pressing, the majority of relatively polar free volatiles are extracted into the juice fraction (*19*). Similarly, bound forms (glycosides) which are relatively polar owing to its sugar moiety are also extracted (*ca.* 70%) (*7*) onto the juice fraction. The composition of glycosides varied greatly among cultivars (*20, 21*). Thus, studies analyzing juice aglycones released both by enzymatic and acid hydrolyses had been attempted for cultivars differentiation. Acid hydrolysis was proven as the more effective way for discrimination (*22*) than enzymatic hydrolysis. During winemaking, release of some aglycones was dependent on yeast selection (*23-25*) caused by enzymatic-driven yeast hydrolysis (*10, 11*) although the amount released accounted only for a small

fraction at the end of alcoholic fermentation compared to the potential amounts that could be released during aging via hydrolysis (*4, 25-33*). Under normal conditions where no cork or other off flavor taints are present, how well can wines age depend greatly on the composition of and the release of the bound volatiles in the wine. In all different disciplinary studies, an analytical tool is always needed to measure the glycoside composition. In the following section, we will discuss 1) analytical techniques for isolation, extraction, and quantification of glycosides and aglycones, 2) viticultural factors on glycoside composition 3) Enological factors including a selection of yeasts and lees aging on glycoside compositions.

Analytical techniques of aglycones and glycosides determination

Analytical interpretation is certainly one of the most critical factors required in any studies and is strongly dependent on the employment of the select analytical technique. The biggest challenge in analysis of glycosides in wine lies on the fact of continuous evolution of glycosides from grape juice through various stages of aged wines. During these stages, many chemical reactions such as oxidation, reduction, hydrolysis, rearrangement, and so forth continue to progress. For instance, linalool was further structurally rearranged to form other aglycones, furan and pyran linalool oxides (*34*) while single aglycone may be derived from more than one precursor such as β-damescenone (*35-37*), vitispirane, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (*29*), guiacol, syringol, and phenol (*7*). Furthermore, most glycoside standards are not

commercially available. Thus, the attempts to structurally identify glycosides are very challenging. As the products of secondary metabolites, most free and bound volatiles are present at a low level (part-per-billion to part-per-trillion). Thus, isolation and quantification could be very challenging as well. With the above mentioned challenges and limitations, it is worth noting that the glycoside analytical techniques that have been proposed so far can only interpret part of the story about how bound volatiles are released and evolve from wine making stages to aging process, and how to effectively predict quality of aged wines by measuring the bound fraction in juice and in young wine. The following sections addressed and discussed various analytical techniques applied in the studies of glycosides. As shown in Figure 1, analysis of glycosides can be performed by 1) isolation and extraction of glycosides, 2) analysis of aglycones released by hydrolysis using GC-MS, 3) Analysis of glycosidic glucose using enzymatic assay, 4) analysis of glycosides via derivatization on GC-MS, 5) direct analysis of glycosides using LC-MS/MS. Prior to the first step, juice is commonly prepared by crushing and pressing grapes either using commercial equipment for large juice sample or laboratory equipment such as a waring blender and a centrifuge.

Isolation and extraction of the glycosides.

Isolation and extraction of glycosides were employed commonly by solid phase extraction (SPE) and rarely by liquid extraction (LE) (*27*). This might be due to a better isolation and extraction options in SPE.

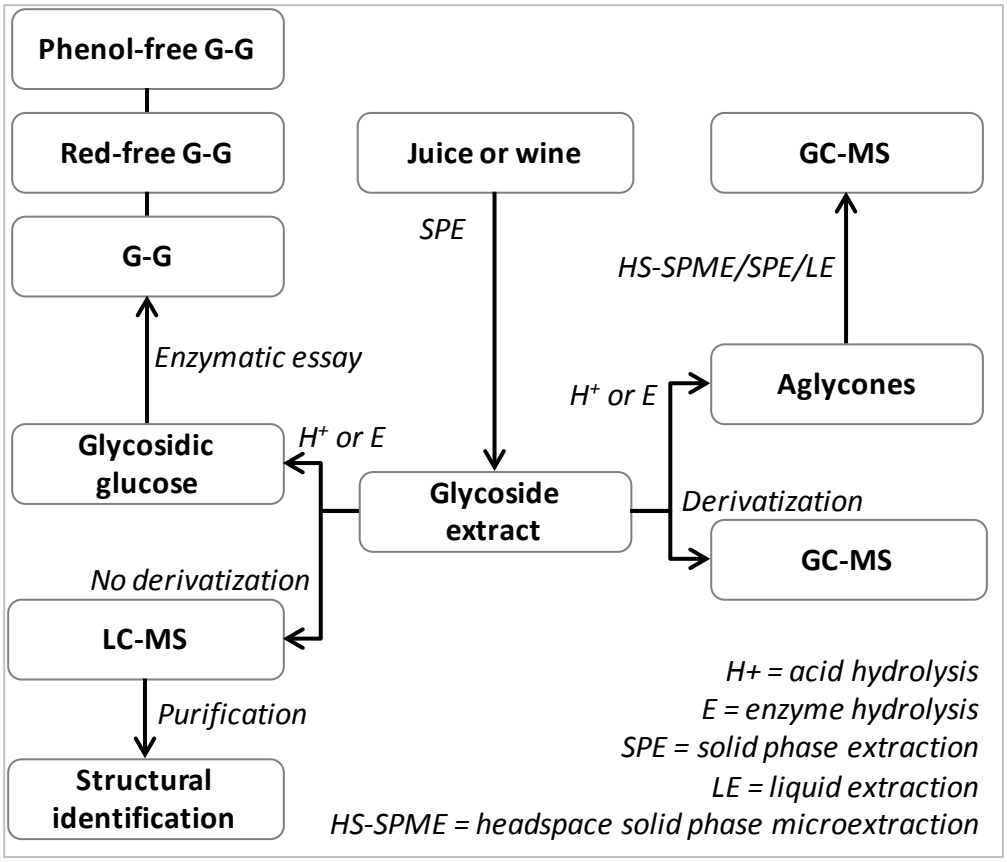

Figure 1 – Various glycoside analytical techniques

The choice of SPE sorbent materials varies greatly among reported studies for glycosides extraction. The most common SPE sorbents used are the silica-based reverse phase C-18 (*27, 38*) and the polymeric resins Amberlite XAD-2 (*15, 39*). Similar to Amberlite XAD-2, employment of another polymeric resin, LiChrolut EN, with a greater capacity of sorbent surface area was relatively new (*40*). By means of measuring the released aglycones of different chemical classes (acids, esters, lactones, benzenes, volatile phenols, vanillin derivatives, terpenes, and norisoprenoids) on GC-MS, a recent study optimizing the application of LiChrolut EN (200 mg) extracted almost two times more than Amberlite XAD-2 (280 mg) and six times more than C-18 resin

(200 mg) (*40*). Although C-18 sorbent was particularly poor in performance on its retention of many different functional groups, norisoprenoids and monoterpenes were best retained on this column (*40*). In addition to the sorbent type, the choice of solvent strength and volume for conditioning, washing, and elution are critical and should be optimized to fit the objective of the study. To date, the only optimization of SPE application for glycoside extraction was noted on a recent study using LiChrolut EN proposing an optimized elution solvent (ethyl acetate:methanol (9:1).

Analysis of aglycones by enzymatic or acid hydrolyses on GC-MS

Both enzymatic and acid techniques attempt to mimic the condition of aged wines thus used for predicting the quality of aroma potential in aged wines. Enzymatic hydrolysis is performed by acceleration of glycosidase activity via enzyme treatment at pH 4-5 while acid hydrolysis is performed by altering the temperature and the pH of juice or wine. This two techniques yielded not only different chemical composition (*27, 41*) but also different sensorial attributes in grape (*22, 42*) and also in malt (*43*). Aglycones released by acid hydrolysis generated more intense fruity and smoky aromas, which were not produced by enzymatic hydrolysis (*42-44*). In a sensorial study, using a duo-trio difference test, aglycones of both enzymatic and acid hydrolyses were significantly differentiated (*42*). Compared to the enzymatic one, acid hydrolytic technique is more effective in distinguishing cultivars and also better at correlating to the aroma of aged wine by means of sensory analysis of the released aglycones

(*22*). Aglycones of various chemical classes released by enzymatic hydrolysis were significantly more abundant compared to those of acid hydrolysis except for the classes of monoterpenes and C_{13} norisoprenoids (*21*). Enzymatic technique might be more efficient in releasing aglycones but less realistic (*28*) since it tends to overestimate especially in the prediction of vinylphenol and vinyl guaicol (*21, 45*). This might be due to impurity of commercial enzymes, which may present a side activity of cinnamate decarboxylase, an enzyme that readily converts cinnamic acid to form vinylphenol (*46*). Furthermore, optimum pH for enzyme activity is usually at pH 4-5 (*11*) which is not realistic for wine's pH. Although acid hydrolysis products generated better sensorial pool of aglycones, it also has its drawback. Unlike enzymatic hydrolysis releasing mainly an intact aglycone, acid-catalyzed hydrolysis is less specific cleaving either the glycosidic linkage (*O*-sugar moiety) to release aglycone or the ether (*O*-aglycone moiety) to yield the carbocation of the aglycone (*27*), which would readily react with other components in grapes via reactions such as acid-catalyzed dehydrations and rearrangements (*47, 48*). Currently, none of these techniques has reached its optimization in predicting the volatiles in finished wine and aged wine (*41*). Thus, the choice of either enzymatic and/or acid hydrolysis should be carefully considered to fit the objective of the study. Besides the common aglycone extraction methods such SPE and LE, HS-SPME can be used for extraction of aglycones or derivatized glycosides (*49, 50*). Comparing SPE and LE for aglycone analysis, SPE showed a better reproducibility than LE (*40*).

Glycosyl-glucose (G-G). As shown in Figure 1, after acid or enzymatic hydrolysis, the glycosidic glucose counterpart can be analyzed by using a HWG-6-PDH enzymatic assay kit *(51).* This method is called glycosyl-glucose or G-G assay. Since hydrolysis application not only releases the D-glucose fraction from aroma precursors but also a great fraction from anthocyanin glycosides in red variety, a modified version of G-G method was introduced by subtracting the anthocyanin molar equivalence from the total GG value (*52*). A few years later, a so-called phenol-free G-G was proposed to further remove any phenol glycosides, which was proposed to have potentially interference with the G-G absorbance reading (*53*). Although G-G method is relatively rapid, it still lacks of specific capability in identifying the types of aglycones or glycosides. Care must be taken considering the incomplete wash off of glucose from grape juice.

Derivatization

Although most studies adopted enzymatic or acid hydrolysis for analysis of aglycones in wine grape and wines, a less popular technique of derivatization using TFA (trifluoroacetylation) (*54, 55*) and TMS (trimethylsilylation) had been reported (*39, 50*) and also been used to identify the types of glycosides (mono-, di-, tri-, or tetra-saccharides) in grapes (*30, 31*). Most derivatization reagents are designed to target certain functional groups such as the hydroxyl group, carboxyl group and so on. Since juice and wine aromas are very

complex consisting of many different types of aromas sharing the same functional group, this technique has the lack of selectivity on the reaction toward the targeting compound(s). Thus, it makes the spectral identification more time consuming and challenging, especially in election ionization mass spectrometry where a molecular weight is not available.

Direct glycoside analysis using LS-MS

Lastly, analysis of glycoside can be performed directly after extraction of glycoside using LS-MS (*7, 49*). There are not many studies reporting the use of this method. Studies that use this method were conducted to identify the chemical structure of the glycosides. Structural identification of glycosides is not straight forward as it involves a purification stage and requires a pure chemical standard for verification. Since most glycoside standards are not commercially available, verification process might be challenging.

Viticultural Factors

Many attempts in viticultural studies on glycoside composition were aimed to cultivate cultivars with abundance of glycosides, especially the monoterpene and C_{13} norisoprenoid glycosides. In reality, it is very challenging to control the yearly reproducibility of the aroma composition in grape. This is mainly due to the variation of uncontrollable seasonal macroclimate. However, alteration of grapevine microclimate such as shoot-thinning and leaf removal practices is regularly performed to attain desirable canopy structure. Basal leaves removal

showed no impact on the sum of free and bound monoterpenes in Riesling (*56*) while other proposed removal on fruiting zone resulted in higher glycosides by means of measuring phenol-free G-G level (*57*). Nitrogen fertilization showed no impact on monoterpene profile (*58*) but significant difference on bound aromas of aged Riesling (*59*). Water deficit was reported to increase phenol-free G-G level (*60*) and bound monoterpenols and C_{13} norisoprenoids (*61*). Bound TDN and Vitispirane were reported to be higher in warmer growing regions (South Africa) and lower in colder ones (Germany) (*62*) while monoterpenes were reported to be the opposite (*63*). Compared to the shaded ones, sun-exposed clusters were associated with higher TDN and Vitispirane, released by acid-hydrolysis in South African Riesling (*64*). The timing of the exposure was reported to be pivotal showing optimal at 33 days post-veraison (*65*). Application of gibberellic acid which significantly reduced the yield (clusters per vine) did not significantly alter the glycoside composition (by G-G assay) for a consecutive two-year study (*66*). When observing various training systems such as vertical shoot positioning, Smart-Dyson, and Geneva double curtain, the later had the highest level of phenol-free glycosides (*67*). Since different training systems provide different levels of vine vigor, canopy microclimate, sunlight interception, and crop yield, it's not clear which of these levels has altered the composition of glycoside in Geneva double curtain training system. Although all of these viticultural studies showed impact on glycoside composition, a direct comparison between and among studies

should be carefully evaluated as these studies had applied various analytical techniques.

Enological factors

Must clarification

A study observed impact of must clarification techniques – a natural settling, a natural settling with addition of pectinase with and without filtration, a natural settling with addition of pectinase then followed by treatment of fining agents: bentonite, casein, silica gel, and activated charcoal – on glycoside composition by measuring the G-G concentration. Results suggested that any of these techniques significantly decreased glycosides of linalool, geraniol, benzyl alcohol, 2-phenylethanol, and eugenol while a natural settling without any further treatments had the least impact in glycoside reduction (*68*).

Yeast selection

During winemaking, release of some aglycones was dependent on yeast selection (*23-25*) by enzymatic-driven yeast hydrolysis (*10, 11*) although the amount released was accounted only for a small fraction compared to the potential amounts that could be released during aging via hydrolysis (*4, 25-33*). Not all glycosides were transformed into aglycones by yeast (*69*). Clearly, yeast selection has a significant impact on aroma profile in wines. This is well documented in any fermentation guidebook published by yeast suppliers and has been supported by numerous studies (*11, 70-72*). Clearly, yeast selection is one of the critical steps in winemaking unless the route of spontaneous or

wild fermentation by native wild yeasts and other microorganisms is preferred. In research, β-glucosidase activity of yeast has been a specific interest due to its metabolic capability in hydrolyzing glycosides to release their corresponding aglycones during fermentation. Sarry et al. published a comprehensive review covering both endogenous and exogenous glycosidases along their enzymatic mechanism and activities in releasing volatiles from the glycosylated fractions (*73*). Numerous studies about screening yeast's β-glucosidase activity have been published covering various species and strains from *Saccharomyces* (*74, 75*), *non-Saccharomyces* species such as *Debaryomyces and Brettanomyces* (*76-78*), and other genera (*79, 80*). Of hundreds of yeast species screened in multiple studies, *Saccharomyces cerivisiae, Saccharomyces bayanus, Brettanomyces bruxellensis, Hanseniaspora uvarum, Kloeckera apiculata, Torulaspora delbrueckii and Debaryomyces carsonii* were proposed to have a high level of glycosidase activity and were selected for evaluating their abilities in releasing aglycones (*11*). Detailed information about its activity strength at pH 5.0 along with the type of strains were reported by Hernández-Orte P. et al. (*11*). In addition to yeast, β-glucosidase activity of lactic acid bacteria, *Oenococcus* oeni, has also been reported (*76, 78*). Although this bacteria expressed β-glucosidase activity against substrate *p*-nitrophenyl-β-D-glucopyranoside in synthetic juice or wine fermentative medium (*76, 78, 81-83*), no activity was reported against *Vitis vinifera* Viognier grape glycosides (*78*). This might due to the limited α-L-rhamnopyranosidase and α-L-

arabinofuranosidase activities (*83*). Although studies showed *de novo* synthesis of monoterpenes by *Saccharomyces* yeast strains, fermentation was mostly carried out on fermentation medium (*24, 84*). Demonstration on real grape juice is needed to guarantee the effectiveness of treatment.

Fate of glycosides during aging on Lees

Wine aging on lees (yeast biomass produced at the end of an alcoholic fermentation) is a stylistic approach on table wine production, yet it is a required step in sparkling wine production. Traditionally, the making of sparkling wine requires a second phase of fermentation in a bottle with a prolonged aging on lees (*85*). This second step was believed to increase complexity of aroma profile via yeast-lyase activities: hydrolysis and oxidation (*85*). However, lees aging could potentially contribute a list of fermentative sulfur compounds such as ethylmercaptan, dimethyl sulfide, diethyl sulfide, dimethyl disulfide (*86*) which were associated with faulty aromas (*87*). How bound volatiles evolve during lees aging had been the focus in some studies (*88-90*). A study measuring the glycosyl-glucose (G-G) fraction had shown a decrease of G-G level on lees aged wines. Whether the decrease was due to hydrolysis of the O-D-glucose linkage or due to the binding affinity of the lees was unknown (*90*). Lees has exhibited binding affinity toward oak-derived volatiles such as eugenol, 4-propylguaiacol, 4-methylguaiacol, furfural, and 5-methylfurfural (*88*). Lees aging over a period of 3 and 9 months showed a decreasing trend on majority of aglycones which suggested the capability of

lees to act as a sorbent or further metabolizing of aglycones (*89*). An evidence of yeast lees' capability in decreasing aroma molecules such as vanillin (*89, 91*) was reported even thought the mechanism of its degradation is unclear.

Can we predict quality of aged wines by measuring the aglycone or glycosides levels in grape juice or young wine?

Comparing sensorial quality of aglycones in wine released by enzyme and acid hydrolyses and the volatiles in the corresponding wine showed a distinct separation on principal component analysis (PCA). The hydrolyzed aromas were separated on the first component (70.9%) from wine aroma while the aromas of the two hydrolytic techniques were differentiated on the second component (20.3 %) (*92*). The other study comparing the aglycones of grape juice and volatiles in the corresponding wine showed also a clear difference in the chemical composition (*41*). Aglycones of enzymatic hydrolysis were differentiated from aglycones of acid hydrolysis and wine volatiles on the first component (29 %). Differentiation between wine volatiles and aglycones of acid hydrolysis was distinctive on the second component (20%). Lastly, another study showed wine volatiles were completely differentiated from aglycones of both acid and enzymatic hydrolysis (*42*). Although the orientation on the PCA component is different, all of these 3 studies showed a distinct separation on the first and second components for the quality and quantity of wine volatiles and aglycones. In conclusion, the current literature methods for analysis of glycosides either by measuring the aglycones or the glucose

counterparts are still not efficient in predicting the glycosides composition or aroma quality in aged wines.

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Chapter 5

Profiling acid-hydrolyzed aglycones of monoterpenes, C_{13} norisoprenoids, benzenoids in *Vitis vinifera* L. cv. Riesling during grape maturation

Introduction

A great amount of aromas in wine grapes are present in bound form as being glycosylated to sugar in the form of an odorless and non-volatile glycoside (*1-6*). Thus, glycoside consists of a volatile aroma compound bound to a non-volatile counterpart which can be a mono-saccharide such as β-D-glucopyranoside or di- saccharides such as α-L-arabinofuranosyl-β-D-glucoside, α-L-arabinopyranosyl-β-D-glucoside, β-D-apiofuranosyl-β-D-glucoside, or β-D-xylopyranosyl-β-D-glucoside, α-L-rhamnopyranosyl-β-D-glucoside (rutinoside) (*3, 7-9*). The volatile constituent can be of various aroma classes such as monoterpenes, norisoprenoids, volatile phenols, and vanillin derivatives (*5, 10*). The proportion of glycosides in wine grapes is 2 to 8 fold greater than those present in free form (*6, 11*). During winemaking stage, the volatile constituents are released via yeast metabolism and enzymatic and acid hydrolysis (*12-14*). These released volatiles from their bound glycosidic form are referred as aglycones. It has been reported that only ca. 10% of the potential aromas are released at winemaking stage (cite) and *ca.* 90% of monoterpenes in wine are still present as glycosides (*15*). During barrel and bottle aging, more volatiles or aroma precursors in the form of glycosides will be released via acid hydrolysis at wine pH (*16-20*). With the exception for a

few cultivars such as *Vitis vinifera* Cabernet sauvignon, gewürztraminer and *Vitis labrusca* Concord where their varietal aromas are present in free form in juice, many varietal key odorants are present as glycosides in juice. Thus their varietal characteristic can only be effectively distinguished at wine stage. For example, many key odorants in Riesling described as citrusy, floral and petro-like aromas are highly associated with the chemical compounds of monoterpenes and C_{13} norisoprenoids and are mainly present as glycosides (*21-25*). Thus, studies of glycosides analysis in wine grapes or wines have been of great interest in the past decades. In general, analysis of glycosides can be carried out by 1) quantification of whole glycoside structure which is sugar + aglycone, 2) first cleaving off the glycosidic bond followed by either measuring the released volatiles or measuring the released sugar fraction by derivatization or enzymatic reaction. Unfortunately, due to limited availability of authentic standards, direct whole glycoside structure analysis has been challenging on the identification process. Therefore, most studies in glycosides analysis have been done by measuring aglycones cleaved off by either enzymatic or acid hydrolysis. It is vital to note that enzymatic and acid hydrolysis produce different composition of aglycones (*26-31*). Vitispirane, Actinidiol, β-damascenone, 1,1,6-Trimethyl-1,2-Dihydronaphthalene (TDN), known as the key odorants in aged Riesling (*16-19, 32, 33*), are almost exclusively generated by thermal-acid hydrolysis (*29, 34*). Notably, when heat was applied to treat grape juice (100 °C/15 min) aiming to inactivate endogenous grape enzymes before applying glycosidase enzymes, β-

damascenone and vitispirane and TDN were then detected from enzymatic hydrolyzed fraction (*35, 36*). No study had reported a profiling of monoterpene and C_{13} norisoprenoid and benzenoid aglycones during ripening. While some studies measured exclusively the levels of C_{13} norisoprenoid aglycones during ripening (*37, 38*) , other studies reported levels of monoterpene and/or benzenoid aglycones in *Vitis vinifera* superior seedless (*39*), Muscat Hamburg (*40*), Muscat of Alexandria (*41*) and Muscat of Frontignan (*11*) during ripening. Although a recent study profiled evolution aroma compounds in Riesling from fruit set to harvest, the study focused on the free volatiles and analyzed mainly C6-alchohol and aldehydes (*42*). Since acid hydrolysis has better reproducibility and efficiency in releasing key odorants in Riesling we studied acid-hydrolyzed aglycones of monoterpenes, C_{13} norisoprenoids, and benzenoids during ripening for 2 consecutive seasons.

Materials and Methods

Chemicals

All solvents and aroma standards were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma Aldrich (Allentown, PA), respectively, except for 1,1,6-trimethyl-dihydronaphthalene (TDN) which was synthesized in house. Solid phase extraction (SPE) cartridges packed with 200 mg LiChrolut EN sorbent (Merck, Darmstadt, Germany) were purchased from VWR International (West Chester, PA).

Vineyard specification

In 2007, Riesling (clone 110) on rootstock 3309 was planted with a spacing of 2.75 m between rows and 1.10 m between vines at a Cornell University experimental vineyard in the Cayuga Lake AVA (New York). The experimental vines were pruned to 3 canes per vine with 12 nodes per cane and trained to a Pendelbogen training system (*43*). The experimental site consisted of 3 rows with 20 panels per row and 7 vines per panel.

Weather data collection

Daily temperature and precipitation data from April 1st to Oct 31st were accessed at: http://newa.cornell.edu/index.php?page=all-weather-data. The cumulative growing degree days (GDD) were measured based on 10 °C.

Sample collection and preparation

A panel consisting of 7 vines was treated as a replicate. Four replicate panels were selected from among the 3 rows, and at each sampling time point, 300 g of berries were collected from the middle 3 vines or each replicate. In 2009, 8 sampling points were collected at 2, 7, 21, 23, 28, 44, 49, and 56 days post-veraison (dpv). In 2010, 7 sampling points were collected at -7, 2, 7, 23, 42, 49, 59 dpv. Once collected, samples were kept at -20 °C prior to sample preparation. Using a Waring blender (model no. 5011, Torrington, CT), 200 g thawed berries were blended, loaded into 85 mL-NALGENE polycarbonate centrifuge tubes (VWR International, West Chester, PA), and centrifuged for

30 min at 10,000 rpm and 5 °C (5810 R Centrifuge, VWR International). After centrifuging, the supernatant (juice) was filtered through a No. 41 Whatman filter paper. The supernatant was then subjected to solid phase extraction (SPE).

Glycosides extraction using SPE

A previously described SPE method for glycoside extraction was adopted (*44*). SPE cartridges (12-mL) were manually packed with 1300 mg of LiChrolut EN sorbent (Merck, Darmstadt, Germany) and processed on a Varian 24-cartridge Positive Pressure Manifold (Palo Alto, CA). Prior to sample loading, the cartridges were pre-conditioned with 32.5 mL dichloromethane, 32.5 mL methanol, and 65 mL H_2O. Subsequently, juice prepared above from the 200 g of berries was loaded. After loading, the sorbent bed was washed with 26 mL of H_2O and 40 mL pentane:DCM (2:1 v/v). The retained glycosides were then eluted with 25 mL ethyl acetate:methanol (9:1 v/v). The eluent was first concentrated to *ca*. 5 mL using a Buchi R-210 Rotavapor at 40 °C and *ca.* 170 kPa, then evaporated to dryness under N_2 stream, and finally reconstituted with 10 mL of pH 2.50 citric acid buffer (0.2 M).

Hydrolysis and extraction of aglycones

The reconstituted buffer solution was heated in a 100 °C water bath for 1 h in an encapsulated SPME vial under a N_2-filled headspace. After cooling down to room temperature, the 10 mL buffer was spiked with internal standard

mixtures (prepared in methanol) to yield final levels of 0.2 mg/L 2 octanol for 2009 samples and 0.2 mg/L 4-hydroxy-4-methyl-2-pentanone, 3-ethyl-3-dodecanol, pentanoic acid, 2-octanol, and 2-*sec*-butyl phenol for 2010 samples. After hydrolysis, the released aglycones were isolated by SPE. A pre-packed LiChrolut EN cartridge (200 mg) was preconditioned with 5 mL dichloromethane, 5mL methanol, and 10 mL H_2O before loading the hydrosylate. The cartridge was then dried under a N_2 stream (170 kPa for 20 min) and eluted with 2.8 mL dichloromethane. The eluent was then concentrated to *ca*. 0.3 mL under N_2 prior to analysis by gas chromatography mass spectrometry.

Evaluating contribution of non-glycosylated compounds to aglycone extracts

To evaluate if the water and pentane:DCM (2:1 v/v) washes were effective at removing non-glycosylated compounds, Riesling juice samples were prepared from 56-dpv grapes in 2010, as described above. The SPE-based glycoside extraction was performed on 125 mL samples. Following solvent removal, the sample was reconstituted in citric acid buffer but not heated to minimize acid hydrolysis. The reconstituted sample was then extracted by SPE using the same conditions as described for aglycone extractions.

GC-TOF-MS analysis of aglycones

Aglycones were analyzed on a HP6890 GC (Agilent, Santa Clara, CA) coupled to a Pegasus IV time of flight mass spectrometer (Leco, St Joseph, MI). One

μL was injected, splitless, onto a DB-Wax column (60 m × 0.25 mm × 0.50 μm, Varian, Walnut Creek, CA) connected to a VF-17 ms (1 m × 0.1 mm × 0.2 μm, Varian). Although the system was set up for GCxGC analyses, the GC×GC modulator was turned off during analysis resulting in 1-D GC−TOF-MS. The injector temperature was set to 250 ºC. The purge was opened after 2 min. Helium was used as a carrier gas at a flow rate of 1 mL/min. The temperature program was as follows: initial hold for 1 min at 55 ºC, then increased by 3 ºC/min to 240 ºC, 60 min hold. The secondary column and modulator temperature offset was +15 ºC. The MS transfer line temperature was 260 ºC. The TOF-MS was operated in EI mode with an ionization energy of 70 eV. The electron multiplier was set to 1700 V. MS data from *m*/*z* 20−400 were stored at an effective sampling rate of 5 Hz.

Data processing

Data processing was carried out by Leco ChromaTOF software. Peak identification was performed by NIST library search in combination with literature retention index and/or authentic standard verification. The unique ion, as determined by the ChromaTof software, was used for peak integration. Peak areas for each compound were then divided by the area of an appropriate internal standard. The internal standard mixture covering 4-hydroxy-4-methyl-2-pentanone, pentanoic acid, 2-octanol, 3-ethyl-3-dodecanol, and 2-*sec*-butyl-phenol was used to quantify ketones, fatty acids, monoterpenes, C_{13} norisoprenoids, and benzenoids respectively for 2010

sample. In 2009, we used 2-octanol to semi-quantify intensity of all aroma classes.

Statistical Analysis

Statistical and graphical analyses were performed by JMP version 8 (SAS Institute, Cary, NC).

Results and Discussion

Comparison of hydrolyzed versus non-hydrolyzed samples

Analyzing volatiles of hydrolyzed and non-hydrolyzed samples, we can evaluate the effectiveness of the adopted SPE method in removing any free volatiles from the column. Of a list of 67 aglycones representing various aroma classes, only 20 analytes were found to have ≥1% intensity (non-hydrolyzed analyte's ratio divided by hydrolyzed analyte's ratio) retained on SPE column (Table 1). Most of the short chain fatty acids and alcohols, which are relatively polar analytes, were not effectively washed off. Almost no monoterpenes were detected except for 3,7-dimethyl-1,5-Octadiene-3,7-diol (364 %) and hotrienol (only 3% retained). The high retained fraction of 365% for 3, 7-dimethyl-1,5-Octadiene-3,7-diol could be caused by both ineffective wash off and/or degradation of this analyte during thermal-acid hydrolysis at 100 °C and pH 2.503. It has been reported that this analyte was one of the the most abundance free monoterpenes in *Vitis vinifera* Muscat Hamburg (*40*) and

Table 1 – Evaluating the effectiveness of water and pentane:DCM (2:1 v/v) washes in removing free volatiles retained on the SPE column through comparison of hydrolyzed and non-hydrolyzed samples

No	RI	Name	% retained *
1	1230	(E)- 2-Hexenal,	16%
2	1358	1-Hexanol	47%
3	1412	(E)- 2-Hexen-1-ol,	64%
4	1574	2-methyl-propanoic acid	110%
5	1635	Butanoic acid	62%
6	1854	Hexanoic acid	60%
7	1978	(E)-2-Hexenoic acid,	35%
8	1951	3,7-dimethyl-1,5-Octadiene-3,7-diol	364%
9	1617	Hotrienol	3%
10	1895	Benzyl Alcohol	11%
11	1935	Phenylethyl Alcohol	9%
12	1546	Benzaldehyde	12%
13	1659	Butyrolactone	1%
14	2058	Pantolactone	1%
15	2018	Phenol	51%
16	2097	p-Cresol	25%
17	2220	2-Methoxy-4-vinylphenol	1%
18	2600	Vanillin	9%
19	2865	Homovanillyl alcohol	26%
20	2999	Homovanilic acid	23%

*****Percent of free fraction retained on SPE column was calculated by the peak ratio of non-hydrolyzed sample divided by its hydrolyzed sample.

Muscat of Alexandria (*41*) and was found higher level in free than in bound forms at harvest(*39, 40*). At wine pH, this compound was readily transformed into other monoterpenes such as hotrienol and nerol oxide via acid-catalyzed dehydration (*7*). Thus, it is not surprising to observe a great reduction on hydrolyzed fraction. No C_{13} norisoprenoids were found while a few benzenoids were not washed off but the level was ≤ 26% except for phenol. Although the wash did not effectively remove all free fractions, the current method was not altered as increasing the strength and volume of the wash might remove not

only all free fractions but also the desired glycosides. With this method, we focused on the three major glycoside classes: monoterpenes, C_{13} norisoprenoids, and benzenoids.

Weather conditions for 2009 and 2010

Having higher cumulative growing degree days and total precipitation, 2010 season reflected a warmer and wetter growing condition than 2009. From April 1st to October 31st, the cumulative growing degree days were 1278 GDD in 2009 and 1551 GDD in 2010 while total precipitation was 439 mm in 2009 and 594 mm in 2010 (Figure 1). Observing segmented weather data (berry development stage and berry ripening stage) in Table 2, we noted that the vast difference of cumulative growing degree days was between Apr and Aug during berry cell development stage (1062 GDD in 2009 and 1314 GDD in 2010). The major difference of total precipitation was between Sept and Oct during berry ripening stage (51 mm in 2009 and 199 mm in 2010). Conclusively, 2010 season was warmer than 2009 at the beginning of the season resulting in earlier veraison onset - Aug 11th (2010) and Aug 25th (2009). Toward the end of the season, 2010 season was much wetter than 2009 causing infection of *Botrytis cinerea.* The infection started around 42 dpvand reached *ca.* 50%, classified as *pourri plein* (*45*) by 56 days post-veraison. Noted that only healthy berries (by visual assessment) were collected from the vines at sampling point, thus, the basic juice parameters at 56 dpv showed comparable values between the 2 seasons except for TA –

2010: 21.3 Brix, 7.2 g/L (TA), and pH 3.04 and 2009: 21.1 Brix, 10.7 g/L (TA), pH 3.06.

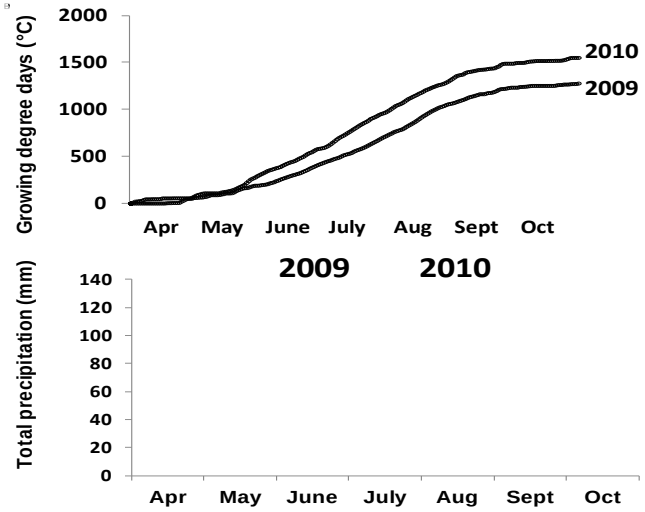


Figure 1 – 2009 and 2010 growing degree days and total precipitation from April 1st to October 31st

Table 2 – Fragmentation of weather data for 2009 and 2010 seasons

Year	Cumulative growing degree days (°C)	Daily mean temperature (°C)	Total precipitation (mm)
	Berry cell development stage (Apr 1st and Aug 31st)		
2009	1062	21.8 (Max), 11.0(Min)	388
2010	1314	24.2 (Max), 12.2 (Min)	395
	Berry ripening stage (Sept 1st and Oct 31)		
2009	216	17.2 (Max), 7.4 (Min)	51
2010	237	17.6 (Max), 8.2 (Min)	199

Behaviors of aglycones during berry ripening

Normalized values (average of 4 field replicates) of aglycone levels during ripening can be found in supplementary Table 1 and 2 for 2009 and 2010 respectively. Figure 2 is the graphical cell plot of the normalized values. Of the 3 major glycoside classes, most monoterpene and all C_{13} norisoprenoid

aglycones reach maximum later in 2010 - around 56 dpv in 2010 versus 44 dpv in 2009. Most benzenoid aglycones reach maximum earlier in 2009 (28 dpv) than in 2010 (56 dpv). In line with other studies reporting increasing pattern for monoterpene (*11, 39-41, 46*) and C_{13} norisoprenoid (*37, 38, 46*) aglycones during ripening, we also observed consistent increasing patterns of these 2 groups in both seasons. Compared to these groups, benzenoid algycones showed less consistent pattern. The volatile phenols: 2-methoxy-4-vinylphenol, syringol, and 2,6-dimethoxy-4-(2-prepenyl)-phenol only showed an increasing trend in 2010 while guaicol only showed a decreasing trend in 2009. Of 8 vanillin derivative aglycones, only homovanilyl alcohol and syringaldehyde showed a consistent increasing pattern in both seasons. Other studies also observed similar inconsistent behaviors of benzyl alcohol and phenylethanol for the 2 years (*39, 47*). Benzyl alcohol aglycone has been reported to decrease gradually during maturation while its free form yielded maximum at ripening (*48*). In Table 3, we calculated the percent increase in 3 maturation segments – early from 2 to 23 dpv, middle from 23 to 44 dpv, and late from 44 to 56 dpv, for aglycones grouped into structural classifications. We noted a distinct difference between acyclic and cyclic hydrocarbon monoterpenes – acyclic monoterpenes increased continuously and sharply during ripening while the cyclic decreased initially followed by slight increases. For all other classifications of monoterpenes, we noted comparable percent increase at each segment with major increase started from 23 days post-

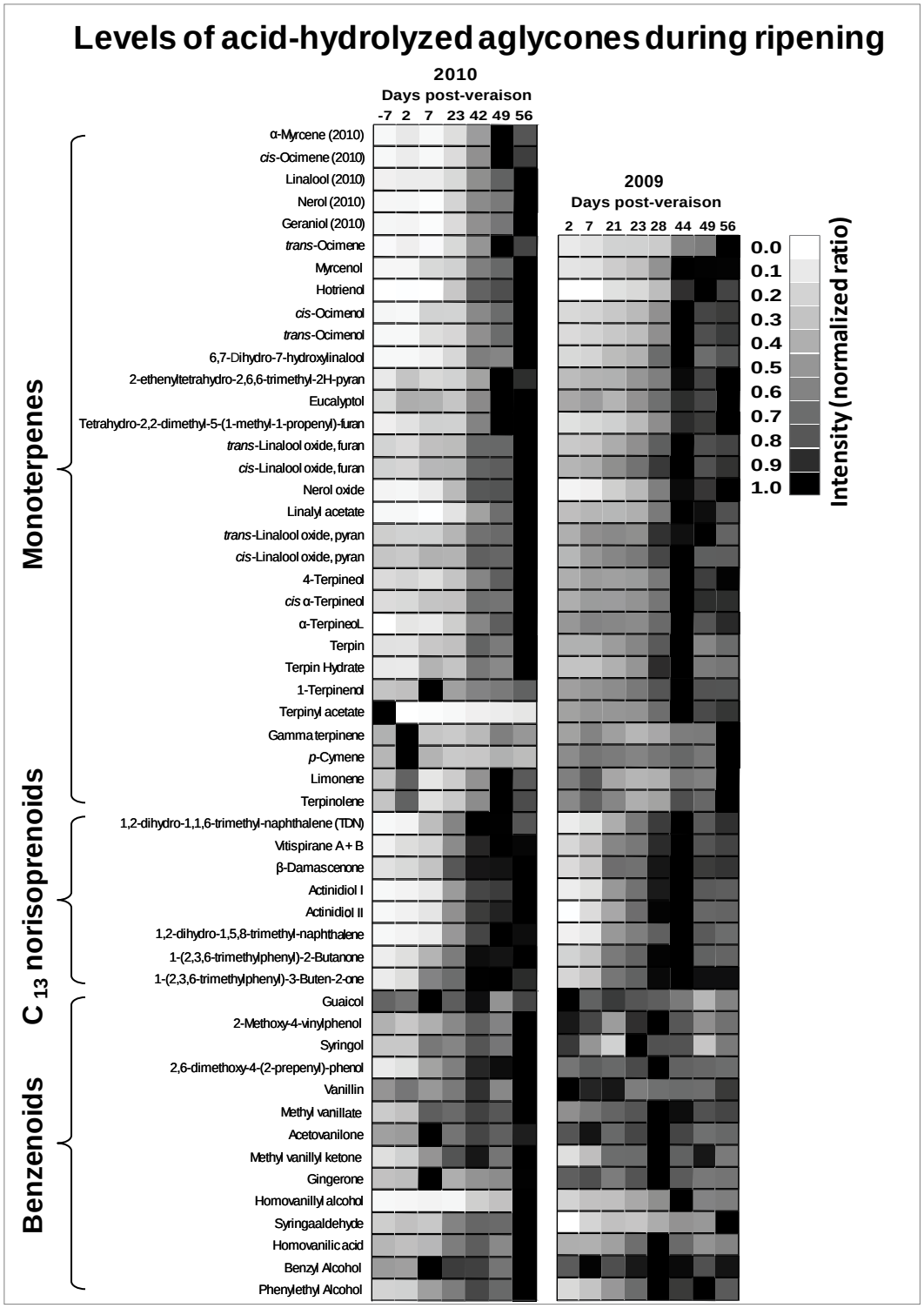


Figure 2 – Levels (normalized to maximum) of acid-hydrolyzed aglycones during ripening in 2009 and 2010 seasons. Average numeric levels of 4 field replicates were available on supplementary Table 1 and 2.

veraison. On the contrary, C_{13} norisoprenoids has majority increase (>50%) by 23 post-veraison. When we observed the glycosylation activity of an isotopic labeled geraniol reported by Luan et al., we noted a major increase after the berries had reached *ca.* 14 Brix (*49*). At 14 Brix, berry ripening stage should be around the middle point between harvest and veraison, which is around 28 days post-veraison.

Behavior of monoterpene algycones

As shown in Figure 2, five monoterpenes (linalool, nerol, geraniol, *cis*-ocimene, and α-myrcene) were not detected in 2009. When comparing enzymatic and acid hydrolyzed products, linalool, nerol and geraniol were more efficiently released by enzymatic hydrolysis as these compounds were noted to be either detected at a low level or not detectable by acid hydrolysis (*26, 30, 31, 50*). These analytes had been reported to rearrange under acidic condition (*7, 51*). We also observed linalool being degraded or rearranged, under thermal-acid treatment (data not shown). Therefore, in accordance to the result in 2009, it was expected that these analytes would not be detected using the current thermal-acid hydrolytic method. However, these analytes were detected in 2010 sample. It's not clear whether their generations were induced by *B. cinerea* infection or by other growing parameters such as excessive amount of rain in 2010. Tracking the behavior of α-terpineol level during ripening, studies employing enzymatic hydrolysis have reported either a constant level during ripening *(39, 40)* or an increase from veraison to

Table 3 – Behaviors of aglycones of various chemical classes during ripening in 2009 and 2010 seasons

		2009 - days post-veraison			2010 - days post-veraison		
Aroma descriptors	**Aroma classes**	**From 2 to 23**	**23 to 44**	**44 to 56**	**From -7 to 23**	**23 to 42**	**42 to 56**
Floral	Acyclic hydrocarbon monoterpenes	+12% NA	+36% NA	+42% NA	+13% *	34% *	35% *
Floral	Cyclic hydrocarbon monoterpenes	-11% ns	+27% *	+29% *	-8% *	+17% ns	+19% *
Citrusy/floral	Acyclic monoterpenols	+19% *	+67% *	-10% *	+16% *	+39% *	+42% *
Floral	Cyclic monoterpenols	+12% *	+46% *	-16% *	+18% *	+32% *	+32% *
Minty/floral	Oxygenated monoterpenes	+20% *	+47% *	-7% ns	+14% *	+35% *	+35% *
Cooked fruit/petrol-like	C_{13} norisoprenoids	+56% *	+32% *	-18% *	+54% *	+33% *	+3% ns
Smoky	Volatile phenols	+2% ns	-14% *	-10% ns	+26% ns	+24% *	+9% ns
Vanilla/woody	Vanillin derivatives	+17% ns	+14% *	-4% ns	+28% *	+16% *	+26% *

The symbols * and ns indicated significant difference and no significant difference respectively by paired t-test of the 2 time points, $p<0.05$). NA indicated that statistical analysis is not available as the class consisted of only *trans*-Ocimene in 2009 sample.

Acyclic hydrocarbon monoterpenes *trans*-Ocimene, *cis*-Ocimene (2010), α-Myrcene (2010), **cyclic hydrocarbon monoterpenes:** Gamma terpinene, *p*-Cymene, Limonene, Terpinolene**. Acyclic monoterpenols:** Hotrienol, Myrcenol, 6,7-Dihydro-7-hydroxylinalool, *cis*-Ocimenol, *trans*-Ocimenol (2010), nerol (2010), geraniol (2010), linalool (2010). **Cyclic monoterpenols** α-TerpineoL*, cis* α-Terpineol, 4-Terpineol, 1-Terpineol, Terpin, Terpin hydrate**. Oxygenated monoterpenes**: 2-ethenyltetrahydro-2,6,6-trimethyl-2H-pyran, Tetrahydro-2,2-dimethyl-5-(1-methyl-1-propenyl)-furan, Eucalyptol, Linalyl acetate, Nerol oxide, *cis*- & *trans*-Linalool oxide, furan*, cis*- & *trans*-Linalool oxide, pyran. **C_{13} norisoprenoids**: TDN, Vitispirane A + B, β-Damascenone, Actinidiol I & II, 1,2-dihydro-1,5,8-trimethyl- naphthalene, 1-(2,3,6-trimethylphenyl)- 2-Butanone, 1-(2,3,6-trimethylphenyl)- 3-Buten-2-one. **Volatile phenols**: Guaicol, 2-Methoxy-4-vinylphenol, Syringol, 2,6-dimethoxy-4-(2-prepenyl)-phenol. **Vanillin derivatives:** Vanillin, Acetovanilone, Gingerone, Methyl vanillate, Methyl vanillyl ketone, Syringaaldehyde, Homovanilic acid, Homovanillyl alcohol.

ripeness (*11*) where the increase was not as sharp as what we have observed for the 2 seasons. Similar to our finding, most studies reported an increase of linalool during ripening (*11, 40, 41*) while one reported a constant level (*39*). Although free geraniol and eucalyptol in Riesling existed from low level to not detectable during ripening (*42*), their bound forms shows continuous increase during ripening in 2010. Stevens et al reported transformation of linalool and geraniol to form α-terpineol under acidic condition (cite), it's likely that the shaper increase we observed might be due the contribution from the transformed linalool and geraniol. To compare the data of the 2 seasons, we noted that 2010 level, the season where linalool and geraniol were detected, has much greater increase than that of 2009. Comparing α-terpineol released by acid and enzymatic hydrolysis, the level in acid hydrolysis was always higher than that of enzymatic hydrolysis observed in both red and white varieties (*26, 30*).

Behavior of C_{13} norisoprenoids

As 80% of Riesling acetal was reportedly converted to form TDN after 60 days at pH 3.0 (*52*), it was not surprising that no Riesling acetal was detected in acid-hydrolyzed aglycones (Figure 2). However, its presence by tentative identification had been reported in other white varieties such as Chardonnay and Muscat (*26*) and red variety Nero d'Avola (*53*) with the same hydrolytic technique. Previous studies reporting behavior of C_{13} norisoprenoid aglycones

during ripening used enzymatic hydrolysis (*37, 38*). Since C_{13} norisoprenoid aglycones released by enzymatic and acid hydrolyses were very different (*26, 28, 29, 31*) direct comparison of the specific aglycone is not suitable.

Differences in behaviors of monoterpene and c_{13} norisopreniod aglycones during ripening

Plotting the levels of monoterpene and C_{13} norisoprenoid aglycones during ripening as shown in Figure 3, we noted 2 major differences – the difference on the onset accumulation and the difference between the 2 seasons during harvest time. Although both classes showed a consistent increasing pattern, the onset of significant accumulation and the peak point were different. Monoterpenes started accumulation later and reached the maximum later than C_{13} norisoprenoids. Prior to 23 dpv, no significant accumulation (p>0.05 Anova) was noted in monoterpenes while a significant increase in C_{13} norisoprenoids was noted as early as 7 dpv similar to previous report (*38*). Although both monoterpenes and C_{13} norisoprenoids were synthesized from isopentyl diphosphate in the plastid, their specific synthases are different: geranyl diphosphate synthase (GPP) for monoterpenes and geranygeranyl diphosphate synthase (GGPP) for C_{13} norisoprenoids (*54*). During fruit ripening, C_{13} norisoprenoid accumulation has been proven to be derived from carotenoid degradation via ^{13}C marker study (*37*). The carotenoid cleavage dioxygenase ($VvCCD_1$) gene has also been characterized and expressed showing the capability to cleave carotenoids and lead to production of C_{13} norisoprenoids (*38*). The study noted a one-week delay between the onsets of

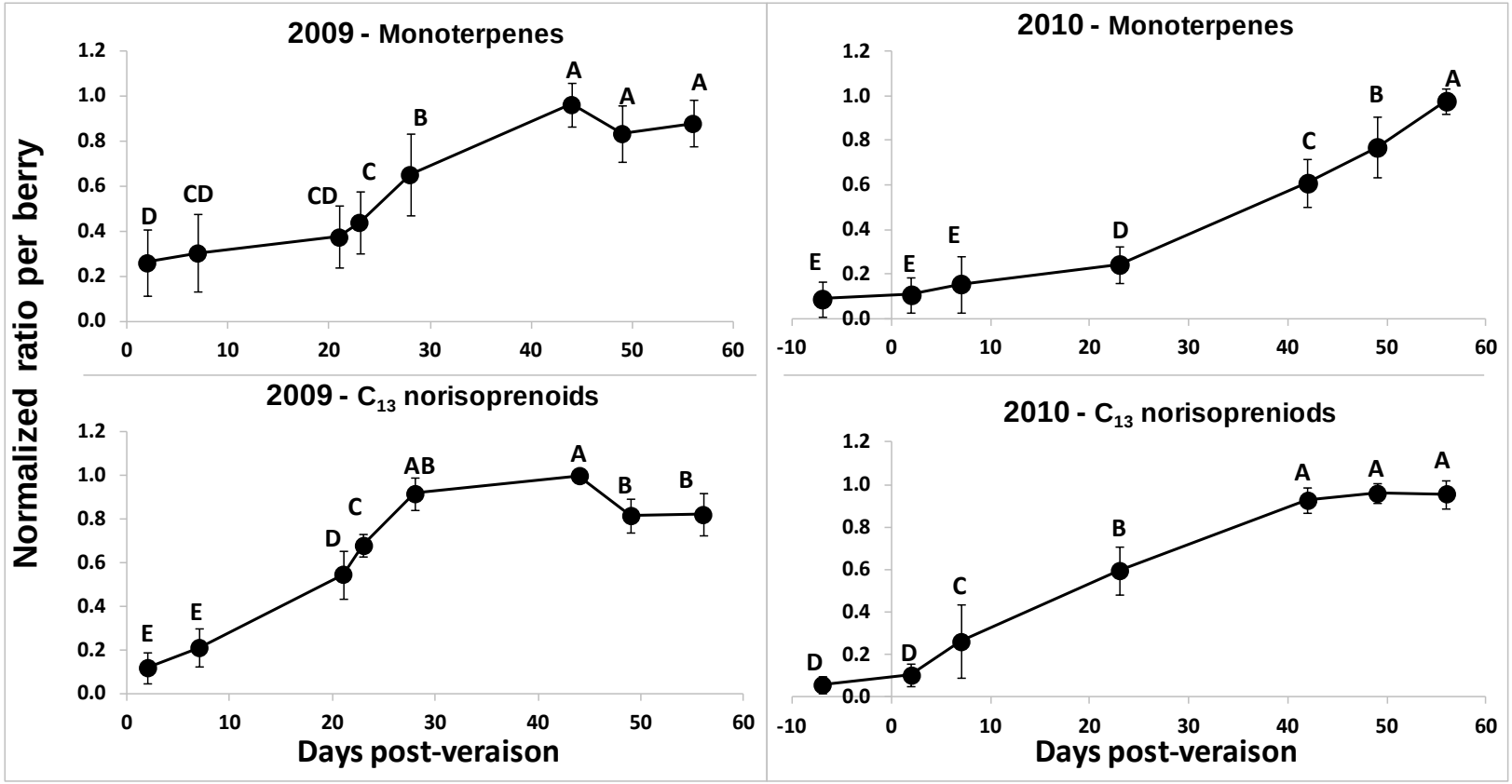


Figure 3 –Behaviors of select monoterpene and C_{13} norisoprenoid aglycones during grape ripening in 2009 (left) and 2010 (right) seasons. The response of normalized ratio was the average of aglycones' intensity within an aroma class. Thus, standard errors represent standard deviations among analytes. Significant difference was analyzed by Anova, $p<0.05$. **Monoterpene aglycones**: 2-ethenyltetrahydro-2,6,6-trimethyl-2H-pyran, *trans*-Ocimene, Tetrahydro-2,2-dimethyl-5-(1-methyl-1-propenyl)-furan, *trans*- & *cis*-Linalool oxide, furan, *trans*- & *cis*-Linalool oxide, pyran, Nerol oxide, Linalyl acetate, Myrcenol, Hotrienol, 4-Terpineol, cis α-Terpineol, *trans*- & *cis*-Ocimenol, α-TerpineoL, 6,7-Dihydro-7-hydroxylinalool, Terpin, Terpin Hydrate, additional 5 monoterpenes only found in 2010 (α-Myrcene, cis-Ocimene, Nerol, Geraniol, Linalool). **C_{13} norisoprenoid aglycones:** TDN, Vitispirane A + B, β-Damascenone, Actinidiol I & II, 1,2-dihydro-1,5,8-trimethyl- naphthalene, 1-(2,3,6-trimethylphenyl)- 2-Butanone, 1-(2,3,6-trimethylphenyl)- 3-Buten-2-one.

$VvCCD_1$ induction and the accumulation of C_{13} norisoprenoids reporting the onset of significant induction of $VvCCD_1$ took place a week prior to veraison. Another study measuring linalool synthase also noted a delay between the induction of linalool synthase and the production of free linalool (*55*). When observing the C_{15}, the authors also noted the later generation of terpenes with respect to expression of synthase (*56*).The timing delay between the product and the synthase induction have been suggested to possibly due to glycosylation of the free volatile. Sandrine et al suggested that, unlike other secondary metabolites, C_{13} norisoprenoids accumulates earlier because of their involvement in grapevine development (*38*). The behaviors of the 2 classes around harvest time were different between the seasons – continuous increase after 40 dpv was noted only in 2010. In 2009, C_{13} norisoprenoids started decreasing after 44 dpv while the level remained constant in 2010. The levels of monoterpenes continuously increased in 2010 and reached plateau in 2009 after 44 dpv.

Impact of B.cinerea on aroma precursors

The growing conditions of the 2 seasons were very different – more heat and rain in 2010 than in 2009. Furthermore, 2010 vines had significant *B. cinerea* infection (*ca.* 50% by 56 dpv) while none was noted in 2009 vines. Comparing the 2 seasons, we have noted the difference on monoterpene and C_{13} norisoprenoid aglycones during harvest time (42 to 56 dpv). For both classes, the levels of aglycones increased in 2010 season (Figure 3). We also noted

most 2010 benzenoid aglycones increased and reached maximum at 56 dpv (Figure 2). These increases should not be accounted for the aroma concentration due to Botrytis-induced dehydration since Brix levels and berry weight of the 2 seasons are comparable despite of more skin damage in 2010 berries. As what Thibon et al suggested that although *B. cinerea* infection might not directly generate aroma production, its presence might simulate grape metabolic pathway resulting on the increase of bound fraction (*57*). They observed 100-fold increase of the level of cysteinylated precursor on Botrytis grapes in one week (*57*). Since monoterpenes possess antifungal properties against *B. cinerea* (*58-60*) the presence of botrytis may induce production of free monoterpenes in the vines which may be further glycosylated due to the toxicity. For instance, glutathione-S-conjugate found in wine grapes, had been reported to be able to conjugate toxic compounds (*61, 62*). As the current glycosides extraction method showed good efficiency in washing off free volatiles in particular for the monoterpenes and C_{13} norisoprenoids, it is unlikely that the increase was partly generated from possible higher level of free volatiles from Botrytis infected sample. Furthermore, Amarone wines made of *B. cinerea* infected grapes had shown only minor increase on select free monoterpenes, C_{13} norisoprenoids, and benzenoids volatiles (*63*). Study has shown the botrytis infection changed the enzymatic activities in berries for having lower β-glucosidase and higher α-arabinose and α-rhamnosidase (*64*). Since we employed acid hydrolysis, it was unlikely that the increase was due to the change in enzymatic activities.

Multivariate analysis: pair wise correlation

Noticing many aglycones showed similar patterns during ripening, we conducted multivariate analysis to observed the correlation among aglycones. Table 4 to 7 showed pair-wise correlations of the 3 major classes for the 2 seasons. Each table presented superimposed correlation values of 2010 and 2009 – above r=1 is the correlation of 2010 data and below r=1 is the correlation of 2009 data. Overall, the 2010 data has slightly higher correlation values than 2009 data. This might be due to improvement on using various internal standards in 2010 versus one internal standard (2-octanol) in 2009 as smaller RSD was noted in 2010 data. Table 4 listed select monoterpene aglycones that showed increasing trend during ripening. In 2010, most aglycones, except eucalyptol where the r>0.8, had high pair wise correlation (r>0.9). Eucalyptol found in aged Riesling at minor quantity could be produced from linalool and geraniol via acid catalyzed rearrangement (*17*). Hotrienol and nerol oxide, degraded from the odorless 3,7-dimethyl-1,5-Octadiene-3,7-diol (*7*) showed r>0.995 in 2010 and r>0.993 in 2009. Linalool was reportedly transformed to furan and pyran linalool oxides (*65*), we noted correlation between linalool and cis-linalool oxide, furan to be 0.917 in 2010. Similar to other report (*24*), C_{13} nor-isoprenoid aglycones were highly correlated (R>0.90) as shown in Table 6. Most aglycones within benzenoid classes were not well correlated as shown in Table 7. In both seasons, 2-methoxy-4-

Table 4 – Correlations (r) of select monoterpene aglycones that showed increasing trend in 2009 (below **1.000**) and in 2010 (above **1.000**) season.

Analyte		**Analyte reference**												
reference		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1	Nerol	**1.000**	0.997	0.960	0.971	0.946	0.974	0.837	0.950	0.935	0.923	0.817	0.952	0.926
2	Geraniol	n.d.	**1.000**	0.955	0.963	0.929	0.974	0.825	0.941	0.915	0.903	0.813	0.938	0.909
3	Linalool	n.d.	n.d.	**1.000**	0.982	0.954	0.992	0.930	0.979	0.938	0.945	0.917	0.984	0.977
4	Myrcenol	n.d.	n.d.	n.d.	**1.000**	0.963	0.976	0.902	0.995	0.961	0.972	0.882	0.974	0.967
5	Hotrienol	n.d.	n.d.	n.d.	0.976	**1.000**	0.941	0.904	0.945	0.995	0.968	0.842	0.967	0.970
6	Linalyl acetate	n.d.	n.d.	n.d.	0.966	0.942	**1.000**	0.899	0.971	0.922	0.929	0.893	0.977	0.960
7	*trans*-Ocimene	n.d.	n.d.	n.d.	0.884	0.858	0.808	**1.000**	0.908	0.892	0.882	0.947	0.935	0.934
8	*cis*-Ocimenol	n.d.	n.d.	n.d.	0.975	0.948	0.937	0.854	**1.000**	0.943	0.970	0.897	0.972	0.967
9	Nerol oxide	n.d.	n.d.	n.d.	0.981	0.930	0.940	0.864	0.940	**1.000**	0.973	0.824	0.957	0.963
10	*cis*-Linalool oxide, furan	n.d.	n.d.	n.d.	0.932	0.857	0.916	0.758	0.906	0.971	**1.000**	0.860	0.959	0.968
11	Eucalyptol	n.d.	n.d.	n.d.	0.922	0.865	0.898	0.882	0.929	0.903	0.867	**1.000**	0.913	0.919
12	4-Terpineol	n.d.	n.d.	n.d.	0.958	0.917	0.946	0.881	0.957	0.930	0.894	0.962	**1.000**	0.989
13	α-TerpineoL	n.d.	n.d.	n.d.	0.809	0.703	0.841	0.710	0.764	0.834	0.863	0.840	0.875	**1.000**

A complete data set (4 field replicates of all time points) displayed on a scattered plot is available in supplementary Figure 1A and 1B 2009 and 2010 respectively. The symbol n.d. indicates that these analytes (nerol, geraniol, linalool) were not detected in 2009 season.

Table 5 – Correlations (r) of hydrocarbon monoterpene aglycones in 2009 (below **1.000**) and in 2010 (above **1.000**) season.

	α-Myrcene	*cis*-Ocimene	*trans*-Ocimene	Gamma terpinene	*p*-Cymene	Terpinolene	Limonene
α-Myrcene	**1.000**	0.995	0.997	0.222	-0.186	0.833	0.821
cis-Ocimene	n.d.	**1.000**	0.999	0.187	-0.221	0.815	0.800
trans-Ocimene	n.d.	n.d.	**1.000**	0.189	-0.220	0.816	0.800
Gamma terpinene	n.d.	n.d.	0.879	**1.000**	0.903	0.710	0.720
p-Cymene	n.d.	n.d.	0.765	0.878	**1.000**	0.365	0.377
Terpinolene	n.d.	n.d.	0.791	0.944	0.765	**1.000**	0.996
Limonene	n.d.	n.d.	0.672	0.912	0.736	0.930	**1.000**

A complete data set (4 field replicates of all time points) displayed on a scattered plot is available in supplementary Figure 2A and 2B 2009 and 2010 respectively. The symbol n.d. indicates that these analytes (α-myrcene and *cis*-Ocimene) were not detected in 2009 season.

Table 6 - Correlations (r) of C_{13}norisoprenid aglycones in 2009 (below **1.000**) and in 2010 (above **1.000**) season.

		Analyte reference							
Analyte reference		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	1,2-dihydro-1,1,6-trimethyl-naphthalene	**1.000**	0.956	0.920	0.910	0.918	0.942	0.969	0.944
2	Vitispirane A + B	0.983	**1.000**	0.922	0.971	0.973	0.978	0.975	0.900
3	β-Damascenone	0.886	0.906	**1.000**	0.939	0.941	0.937	0.926	0.912
4	Actinidiol I	0.954	0.954	0.955	**1.000**	0.999	0.979	0.955	0.877
5	Actinidiol II	0.931	0.928	0.927	0.986	**1.000**	0.983	0.959	0.884
6	1,2-dihydro-1,5,8-trimethyl-naphthalene	0.967	0.970	0.925	0.973	0.938	**1.000**	0.968	0.895
7	1-(2,3,6-trimethylphenyl)-2-Butanone	0.936	0.951	0.854	0.936	0.919	0.948	**1.000**	0.938
8	1-(2,3,6-trimethylphenyl)-3-Buten-2-one	0.954	0.948	0.941	0.959	0.921	0.964	0.924	**1.000**

A complete data set (4 field replicates of all time points) displayed on a scattered plot is available in supplementary Figure 3A and 3B for 2009 and 2010 respectively.

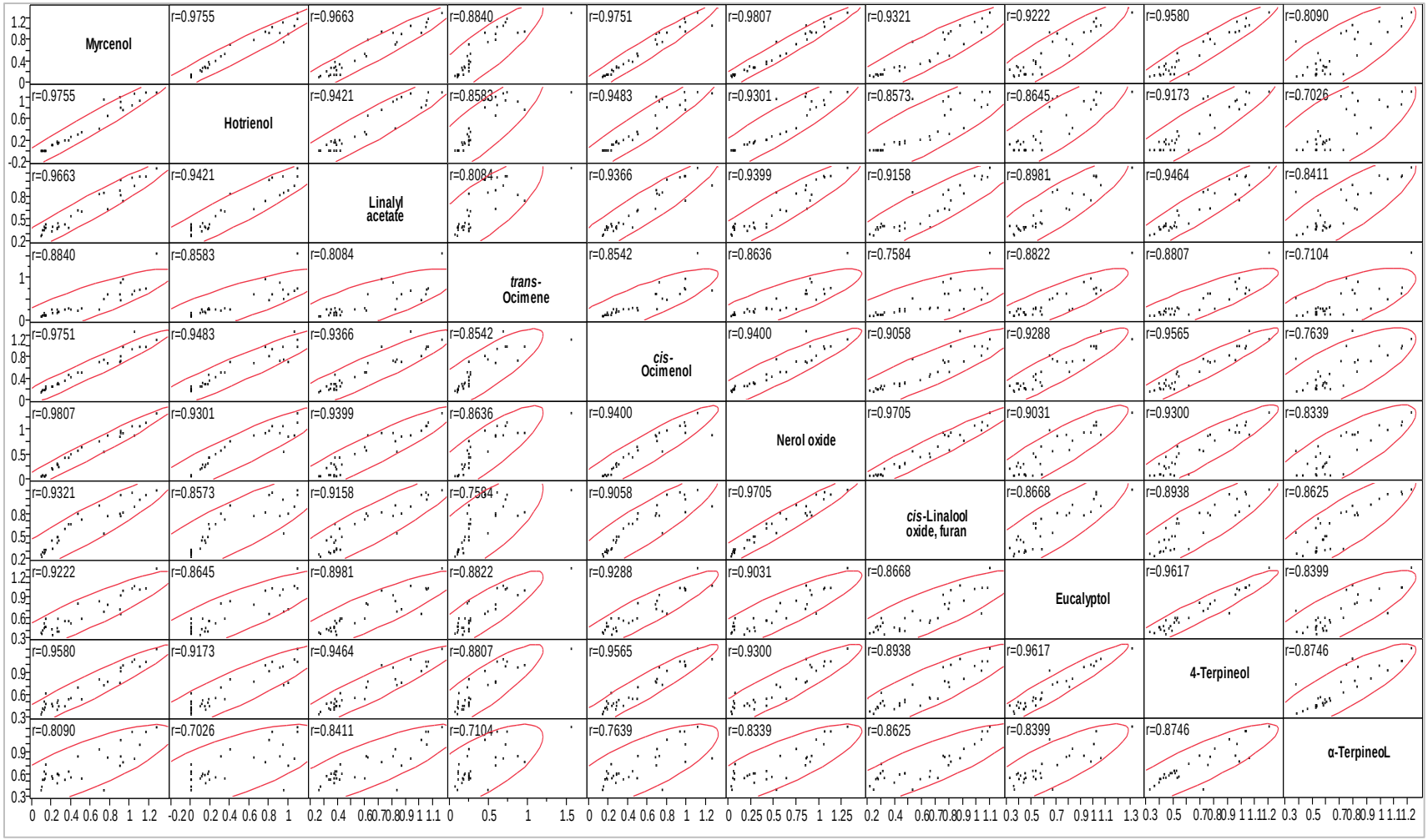
Table 7 - Correlations (r) of benzenoid aglycones during ripening for 2010 (above **1.000**) and 2009 (below **1.000**)

		Analyte No.													
Analyte No.		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	Guaicol	**1.000**	0.291	0.479	0.027	0.283	0.271	0.432	0.273	0.347	0.064	0.059	0.223	0.384	0.068
2	2-Methoxy-4-vinylphenol	0.642	**1.000**	0.866	0.819	0.766	0.791	0.613	0.849	0.460	0.825	0.844	0.935	0.478	0.766
3	Syringol	0.521	0.848	**1.000**	0.834	0.632	0.880	0.792	0.842	0.636	0.683	0.801	0.816	0.685	0.798
4	2,6-dimethoxy-4-(2-prepenyl)- phenol	0.389	0.656	0.486	**1.000**	0.557	0.892	0.673	0.879	0.444	0.629	0.853	0.774	0.570	0.889
5	Vanillin	0.373	0.013	-0.090	-0.094	**1.000**	0.572	0.405	0.660	0.296	0.663	0.756	0.787	0.313	0.591
6	Methyl vanillate	-0.060	0.239	0.265	0.640	-0.280	**1.000**	0.890	0.894	0.703	0.628	0.729	0.723	0.740	0.823
7	Acetovanilone	0.542	0.846	0.599	0.762	0.094	0.446	**1.000**	0.691	0.861	0.478	0.454	0.516	0.743	0.598
8	Methyl vanillyl ketone	-0.303	-0.176	-0.072	-0.389	-0.430	0.770	0.022	**1.000**	0.438	0.606	0.812	0.843	0.666	0.858
9	Gingerone	0.451	0.796	0.629	0.759	-0.005	0.488	0.935	0.146	**1.000**	0.559	0.335	0.344	0.700	0.467
10	Homovanillyl alcohol	-0.234	-0.063	-0.128	-0.204	-0.257	0.721	-0.163	0.417	-0.130	**1.000**	0.743	0.790	0.353	0.658
11	Syringaaldehyde	-0.290	-0.203	-0.060	-0.077	-0.016	0.559	-0.133	0.399	-0.096	0.532	**1.000**	0.854	0.493	0.864
12	Homovanilic acid	0.223	0.540	0.525	0.729	-0.268	0.808	0.543	0.608	0.530	0.468	0.304	**1.000**	0.464	0.780
13	Benzyl alcohol	0.195	0.442	0.283	0.604	0.123	0.626	0.721	0.253	0.726	0.377	0.289	0.431	**1.000**	0.743
14	Phenylethyl alcohol	-0.464	-0.186	-0.054	-0.218	-0.539	0.686	-0.152	0.855	-0.038	0.565	0.532	0.549	0.096	**1.000**

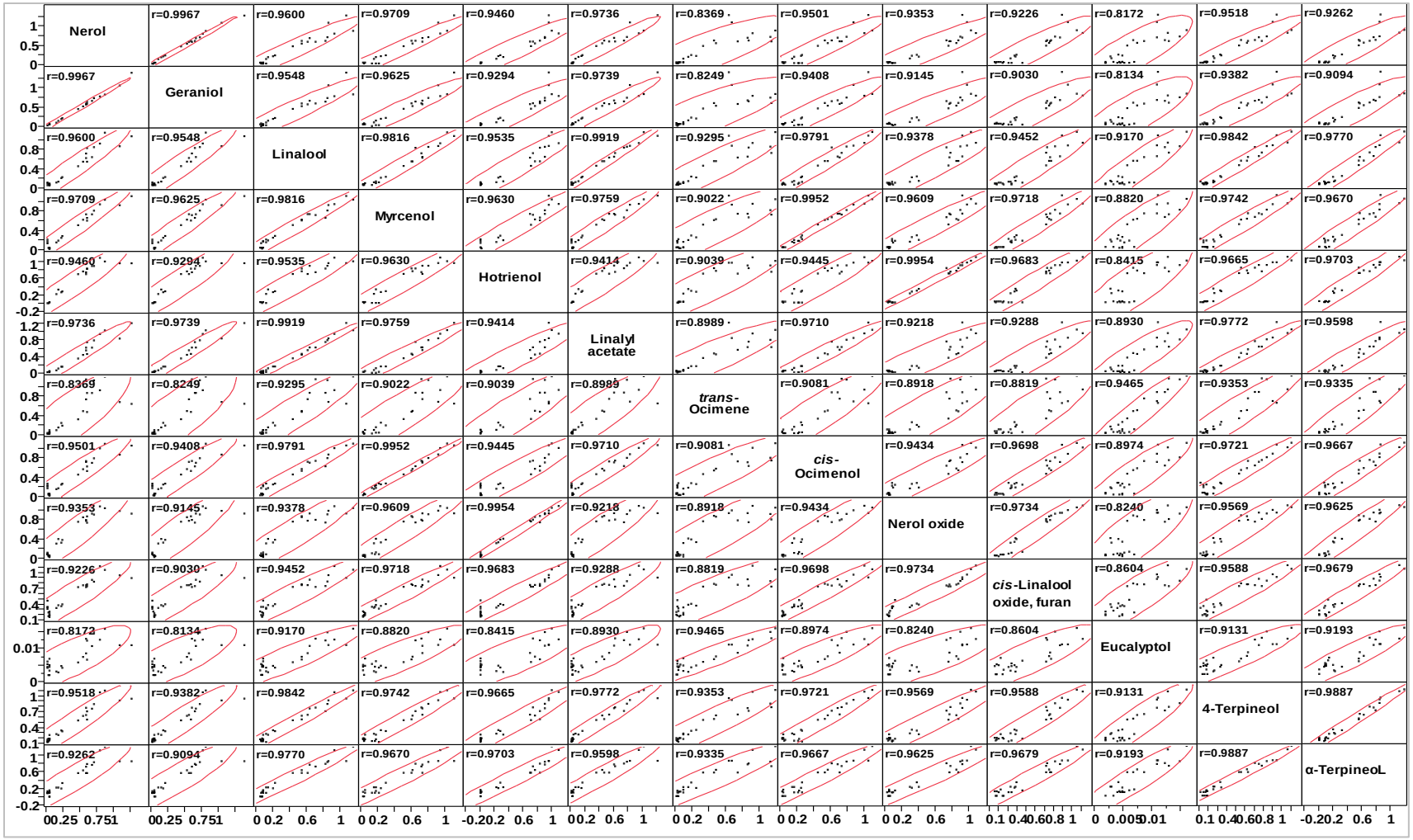
vinylphenol and syringol were well correlated, r>0.8. With these tables showing pair wise correlation values among aglycones, a few key aglycones per aroma class can be used as a proxy to predict aroma potential thus determining the optimum prior to harvesting grapes.

Conclusion

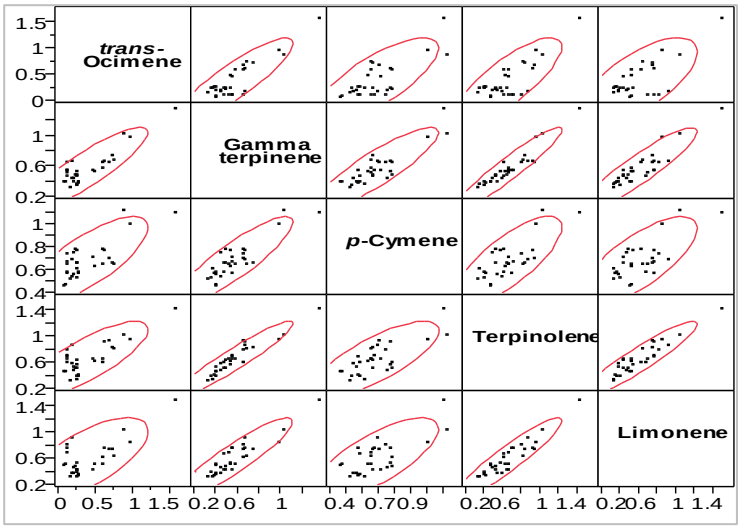
Tracking the behaviors of these aglycones during berry ripening is vital to understand how harvest date can influence Riesling aroma potential and may also provide insights into biochemical pathways underlying glycosides accumulation. Although most monoterpenes and C_{13} norisoprenoids exhibited consistent increasing trend during grape ripening, their major accumulations were different – earlier in C_{13} norisopreniods (>50% prior to 23 dpv) than in monoterpenes. The behavior of benzenoids was inconsistent for the 2 seasons. Within its aroma class, most monoterpene and C_{13} norisoprenoid aglycones were highly correlated (r>0.9). Measuring a few key odorants per aroma class can be used as a proxy to predict aroma potential thus determining the optimum harvest time - no earlier than 44 dpv.



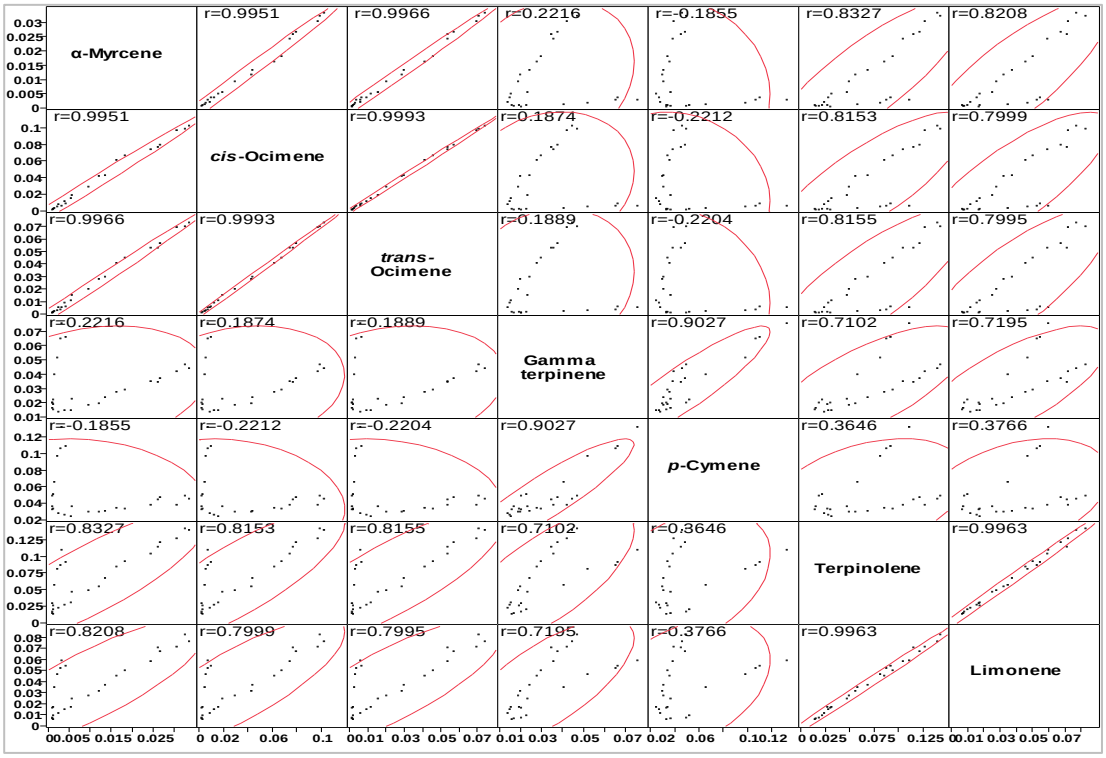
Supplementary Figure 1A – 2009 scattered plot of select monoterpene aglycones that show inreasing trend. Data points are 4 field replicates X 8 time points (2,7, 21, 23, 28, 42, 49, and 56 days post-veraison).



Supplementary Figure 1B – 2010 scattered plot of select monoterpene aglycones that show inreasing trend. Data points are 4 field replicates X 7 time points (-7, 2, 7, 23, 44, 49, and 56 days post-veraison).



Supplementary Figure 2A – 2009 scattered plot of hydrocarbon monoterpene aglycones. Data points are 4 field replicates X 8 time points (2,7, 21, 23,28, 42, 49, and 56 days post-veraison).



Supplementary Figure 2B – 2010 scattered plot of hydrocarbon monoterpene aglycones. Data points are 4 field replicates X 7 time points (-7, 2, 7, 23, 44, 49, and 56 days post-veraison).

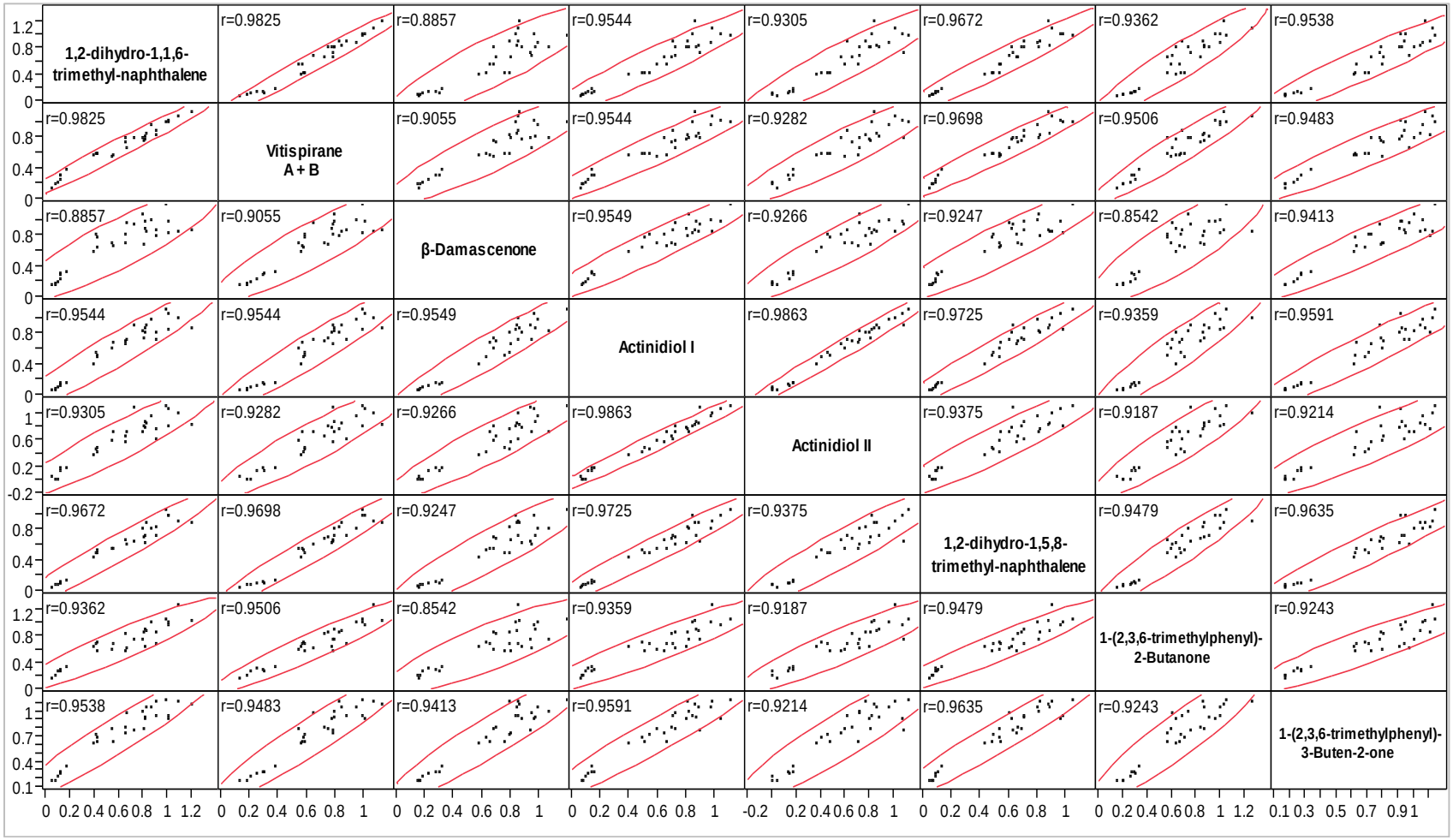


Figure 3A – 2009 scattered plot of C_{13} norisoprenoid aglycones in 2010 season. Data points are 4 field replicates X 8 time points (2,7, 21, 23,28, 42, 49, and 56 days post-veraison).

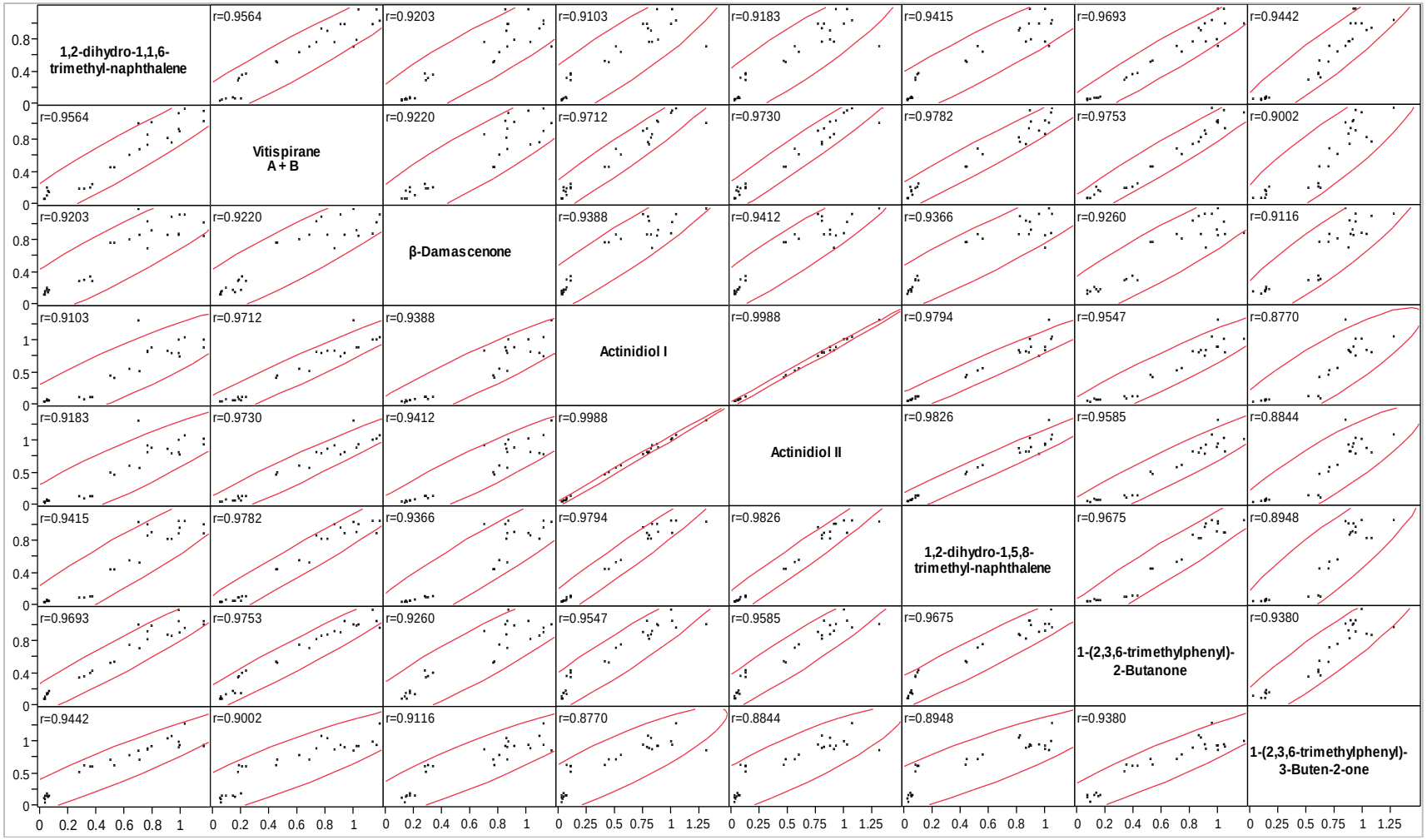


Figure 3B – 2010 scattered plot of C_{13} norisoprenoid aglycones in 2010 season. The data points were 4 field replicates x 7 time-points (-7, 2, 7, 21, 42, 49, and 56 days post-veraison).

Supplementary Table 1- Level of aglycones from various chemical aroma classes during grape ripening in 2009 season

Quant				Days post-veraison[4,5]							
Mass[1]	RI[2]	ID[3]	Analytes	2	7	21	23	28	44	49	56
			Monoterpenes								
139	1131	a	2-ethenyltetrahydro-2,6,6-trimethyl-2H-Pyran	0.337±0.058	0.382±0.044	0.388±0.013	0.496±0.064	0.614±0.124	0.967±0.109	0.859±0.177	1.000±0.254
68	1225	b	Limonene	0.613±0.126	0.794±0.097	0.429±0.074	0.376±0.064	0.396±0.074	0.645±0.189	0.605±0.110	1.000±0.373
111	1242	c	Eucalyptol	0.421±0.076	0.468±0.080	0.378±0.037	0.519±0.059	0.682±0.160	0.908±0.230	0.851±0.187	1.000±0.224
139	1264	a	Tetrahydro-2,2-dimethyl-5-(1-methyl-1-propenyl)-furan	0.134±0.014	0.170±0.029	0.222±0.026	0.349±0.086	0.539±0.127	0.902±0.454	0.878±0.181	1.000±0.274
93	1247	b	*trans*-Ocimene	0.094±0.015	0.126±0.034	0.214±0.041	0.217±0.051	0.245±0.017	0.581±0.117	0.632±0.116	1.000±0.410
93	1272	b	Gamma terpinene	0.447±0.069	0.597±0.073	0.464±0.060	0.370±0.051	0.425±0.057	0.622±0.065	0.630±0.097	1.000±0.290
119	1298	b	*p*-Cymene	0.546±0.098	0.663±0.071	0.630±0.105	0.592±0.106	0.660±0.086	0.724±0.039	0.650±0.012	1.000±0.154
93	1312	b	Terpinolene	0.560±0.116	0.749±0.099	0.577±0.046	0.403±0.062	0.443±0.086	0.839±0.152	0.728±0.107	1.000±0.333
94	1473	b	*trans*-Linalool oxide, furan	0.262±0.042	0.285±0.017	0.418±0.065	0.530±0.116	0.769±0.108	1.000±0.104	0.839±0.231	0.859±0.162
59	1502	b	*cis*-Linalool oxide, furan	0.375±0.065	0.409±0.030	0.536±0.081	0.677±0.121	0.877±0.146	1.000±0.192	0.833±0.258	0.897±0.182
85	1502	b	Nerol oxide	0.047±0.008	0.067±0.004	0.229±0.050	0.351±0.082	0.611±0.113	0.963±0.098	0.890±0.265	1.000±0.210
93	1557	c	Linalyl acetate	0.326±0.066	0.368±0.039	0.362±0.033	0.398±0.025	0.639±0.127	1.000±0.057	0.954±0.121	0.829±0.236
81	1604	b	1-Terpineol	0.454±0.081	0.503±0.101	0.545±0.050	0.645±0.079	0.811±0.136	1.000±0.038	0.822±0.118	0.825±0.070
80	1622	b	Myrcenol	0.115±0.019	0.129±0.014	0.239±0.035	0.312±0.058	0.520±0.122	1.000±0.077	0.983±0.198	0.979±0.215
82	1624	b	Hotrienol	0.002±0.000	0.005±0.001	0.133±0.034	0.163±0.032	0.332±0.063	0.903±0.140	1.000±0.070	0.858±0.192
111	1641	b	4-Terpineol	0.408±0.079	0.475±0.101	0.460±0.025	0.471±0.062	0.642±0.131	1.000±0.079	0.870±0.155	0.979±0.178
93	1661	b	*cis* α-Terpineol	0.409±0.083	0.472±0.094	0.466±0.031	0.513±0.072	0.657±0.121	1.000±0.096	0.908±0.141	0.908±0.170

93	1663	b	*cis*-Ocimenol	0.164±0.023	0.196±0.027	0.262±0.022	0.347±0.075	0.528±0.123	1.000±0.239	0.855±0.155	0.879±0.210
93	1691	b	*trans*-Ocimenol	0.159±0.024	0.197±0.026	0.263±0.021	0.332±0.066	0.530±0.121	1.000±0.256	0.839±0.150	0.881±0.197
59	1732	c	α-TerpineoL	0.494±0.109	0.590±0.096	0.570±0.030	0.552±0.072	0.714±0.195	1.000±0.183	0.810±0.342	0.905±0.217
154	1709	c	Terpinyl acetate	0.445±0.116	0.500±0.114	0.499±0.042	0.514±0.063	0.689±0.163	1.000±0.052	0.847±0.204	0.892±0.163
68	1748	b	*trans*-Linalool oxide, pyran	0.400±0.083	0.533±0.051	0.558±0.080	0.537±0.103	0.893±0.032	0.953±0.027	1.000±0.220	0.741±0.131
68	1767	b	*cis*-Linalool oxide, pyran	0.361±0.110	0.495±0.032	0.576±0.052	0.641±0.091	0.850±0.141	1.000±0.071	0.770±0.184	0.773±0.120
71	1969	b	6,7-Dihydro-7-hydroxylinalool	0.172±0.034	0.196±0.045	0.342±0.072	0.390±0.048	0.704±0.088	1.000±0.041	0.700±0.211	0.824±0.103
81	2117	a	Terpin	0.387±0.077	0.385±0.126	0.477±0.140	0.572±0.101	0.797±0.193	1.000±0.269	0.578±0.229	0.704±0.088
81	2196	a	Terpin Hydrate	0.299±0.065	0.292±0.112	0.396±0.119	0.501±0.098	0.910±0.176	1.000±0.077	0.629±0.203	0.667±0.117
			C_{13} norisoprenoids								
142	1781	c	1,2-dihydro-1,1,6-trimethyl-naphthalene	0.082±0.020	0.134±0.025	0.413±0.012	0.638±0.123	0.874±0.155	1.000±0.012	0.798±0.102	0.896±0.224
119	1553	b	Vitispirane A + B	0.173±0.033	0.304±0.053	0.578±0.014	0.648±0.105	0.901±0.134	1.000±0.009	0.815±0.028	0.860±0.205
121	1852	c	β-Damascenone	0.159±0.016	0.280±0.042	0.693±0.104	0.681±0.019	0.942±0.065	1.000±0.184	0.879±0.132	0.888±0.042
163	1962	b	Actinidiol I	0.077±0.015	0.136±0.020	0.487±0.063	0.672±0.058	0.932±0.058	1.000±0.140	0.794±0.102	0.774±0.073
163	2052	b	Actinidiol II	0.009±0.019	0.154±0.021	0.426±0.049	0.693±0.088	0.982±0.086	1.000±0.163	0.728±0.120	0.742±0.096
157	2044	a	1,2-dihydro-1,5,8-trimethyl-naphthalene	0.065±0.015	0.104±0.017	0.484±0.041	0.610±0.080	0.761±0.121	1.000±0.044	0.795±0.096	0.701±0.126
132	2281	a	1-(2,3,6-trimethylphenyl)-2-Butanone	0.205±0.044	0.299±0.027	0.643±0.058	0.740±0.130	0.978±0.214	1.000±0.049	0.757±0.140	0.739±0.196
173	2349	a	1-(2,3,6-trimethylphenyl)-3-Buten-2-one	0.186±0.027	0.283±0.037	0.658±0.047	0.756±0.085	0.969±0.151	1.000±0.119	0.970±0.166	0.972±0.074

			Benzenoids								
124	1879	c	Guaicol	1.000±0.336	0.755±0.223	0.881±0.477	0.811±0.176	0.777±0.213	0.638±0.204	0.368±0.109	0.600±0.080
108	1894	c	Benzyl Alcohol	0.788±0.120	0.979±0.050	0.840±0.142	0.937±0.097	1.000±0.259	0.966±0.167	0.837±0.126	0.943±0.077
156	1935	c	Phenylethyl Alcohol	0.164±0.024	0.297±0.014	0.489±0.072	0.706±0.018	0.995±0.274	0.883±0.071	1.000±0.208	0.793±0.046
150	2225	c	2-Methoxy-4-vinylphenol	0.938±0.193	0.851±0.131	0.477±0.201	0.903±0.111	1.000±0.210	0.802±0.215	0.529±0.143	0.677±0.054
154	2294	c	Syringol	0.876±0.367	0.502±0.182	0.216±0.018	1.000±0.276	0.835±0.229	0.823±0.330	0.282±0.101	0.641±0.090
194	2566	b	2,6-dimethoxy-4-(2-prepenyl)-phenol	0.665±0.059	0.769±0.095	0.722±0.121	0.846±0.118	1.000±0.251	0.751±0.194	0.729±0.109	0.698±0.029
152	2610	c	Vanillin	1.000±0.256	0.916±0.263	0.940±0.198	0.619±0.128	0.683±0.178	0.703±0.047	0.689±0.154	0.882±0.104
151	2641	b	Methyl vanillate	0.530±0.060	0.650±0.030	0.739±0.082	0.811±0.079	1.000±0.141	0.959±0.220	0.833±0.086	0.855±0.047
151	2682	c	Acetovanilone	0.813±0.121	0.943±0.090	0.720±0.133	0.853±0.130	1.000±0.210	0.855±0.204	0.682±0.102	0.727±0.011
137	2698	a	Methyl vanillyl ketone	0.144±0.026	0.322±0.067	0.701±0.064	0.707±0.123	1.000±0.129	0.757±0.208	0.940±0.289	0.635±0.088
137	2832	c	Gingerone	0.764±0.187	0.838±0.130	0.624±0.116	0.816±0.163	1.000±0.271	0.789±0.184	0.651±0.061	0.667±0.072
137	2875	c	Homovanillyl alcohol	0.202±0.035	0.307±0.030	0.317±0.099	0.423±0.111	0.535±0.123	1.000±0.179	0.589±0.169	0.615±0.248
182	2972	c	Syringaaldehyde	0.000±0.000	0.193±0.008	0.311±0.102	0.284±0.057	0.417±0.115	0.516±0.070	0.483±0.186	1.000±0.141
137	3009	c	Homovanilic acid	0.405±0.086	0.397±0.124	0.461±0.177	0.687±0.088	1.000±0.099	0.717±0.220	0.512±0.091	0.595±0.143

[1]Quant mass was the automatically assigned unique mass by Leco deconvolution software.

[2]Retention index on DB-Wax column

[3]Identification was performed by library search (a) with literature retention index (b) and standard verification (c)

[4]The values reported were normalized to the maximum level during ripening period.

The level of 4 field replicates was acquired by dividing peak area of analyte to peak area of internal standard.

[5]Internal standards: 2-octanol for monoterpenes, 3-ethyl-3-dodecanol for C13 norisoprenoids, 2-secbutylphenol for benzenoids

Supplementary Table 2- Level of aglycones from various chemical aroma classes during grape ripening in 2010 season

Quant				Days post-veraison[5,6]						
Mass[1]	RI[2]	ID[3]	Analytes[4]	-7	2	7	23	42	49	56
			Monoterpenes							
139	1131	a	2-ethenyltetrahydro-2,6,6-trimethyl-2H-pyran	0.097±0.039	0.293±0.121	0.163±0.028	0.210±0.078	0.444±0.047	1.000±0.162	0.907±0.159
93	1175	b	α-Myrcene (2010)*	0.033±0.012	0.103±0.028	0.030±0.005	0.148±0.053	0.467±0.132	1.000±0.135	0.811±0.304
68	1225	b	Limonene	0.299±0.128	0.751±0.072	0.106±0.020	0.243±0.079	0.518±0.137	1.000±0.136	0.795±0.265
111	1242	c	Eucalyptol	0.173±0.057	0.412±0.091	0.388±0.069	0.292±0.075	0.557±0.096	1.000±0.131	0.998±0.187
93	1247	b	*cis*-Ocimene (2010)*	0.025±0.010	0.074±0.024	0.026±0.005	0.167±0.066	0.501±0.172	1.000±0.156	0.856±0.259
139	1264	a	Tetrahydro-2,2-dimethyl-5-(1-methyl-1-propenyl)-furan	0.074±0.050	0.127±0.044	0.221±0.054	0.220±0.061	0.597±0.141	0.992±0.101	1.000±0.142
80	1267	b	*trans*-Ocimene	0.030±0.012	0.080±0.019	0.027±0.006	0.157±0.055	0.525±0.183	1.000±0.151	0.872±0.230
93	1272	b	Gamma terpinene	0.385±0.158	1.000±0.152	0.286±0.050	0.256±0.065	0.352±0.076	0.613±0.092	0.494±0.173
119	1298	b	*p*-Cymene	0.372±0.146	1.000±0.131	0.383±0.082	0.259±0.046	0.279±0.015	0.385±0.048	0.338±0.110
93	1312	b	Terpinolene	0.294±0.117	0.756±0.104	0.136±0.028	0.261±0.085	0.544±0.159	1.000±0.134	0.846±0.273
94	1473	b	*trans*-Linalool oxide, furan	0.202±0.043	0.160±0.034	0.318±0.063	0.340±0.040	0.718±0.056	0.724±0.070	1.000±0.061
59	1502	b	*cis*-Linalool oxide, furan	0.211±0.044	0.187±0.026	0.359±0.066	0.364±0.026	0.746±0.021	0.779±0.071	1.000±0.101
85	1502	b	Nerol oxide	0.047±0.010	0.041±0.012	0.091±0.016	0.347±0.048	0.807±0.057	0.827±0.090	1.000±0.068
71	1554	a	Linalool (2010)*	0.064±0.014	0.080±0.006	0.086±0.024	0.170±0.053	0.535±0.072	0.754±0.100	1.000±0.134
93	1557	c	Linalyl acetate	0.028±0.005	0.034±0.007	0.010±0.002	0.122±0.043	0.449±0.074	0.689±0.087	1.000±0.161
81	1604	b	1-Terpineol	0.292±0.046	0.319±0.057	1.000±0.155	0.459±0.063	0.586±0.057	0.641±0.066	0.756±0.074
80	1622	b	Myrcenol	0.036±0.004	0.036±0.010	0.169±0.034	0.215±0.062	0.610±0.092	0.724±0.061	1.000±0.085
82	1624	b	Hotrienol	0.007±0.002	0.012±0.003	0.002±0.000	0.269±0.042	0.748±0.060	0.840±0.080	1.000±0.065
111	1641	b	4-Terpineol	0.159±0.025	0.195±0.037	0.142±0.024	0.296±0.042	0.584±0.047	0.764±0.055	1.000±0.092
93	1661	b	*cis* α-Terpineol	0.155±0.025	0.172±0.030	0.294±0.050	0.306±0.043	0.685±0.059	0.669±0.054	1.000±0.092
93	1663	b	*cis*-Ocimenol	0.041±0.013	0.032±0.010	0.212±0.037	0.204±0.059	0.562±0.107	0.705±0.073	1.000±0.098

93	1691	b	*trans*-Ocimenol	0.031±0.005	0.033±0.012	0.148±0.032	0.181±0.058	0.531±0.111	0.706±0.083	1.000±0.112
59	1732	c	α-TerpineoL	0.001±0.000	0.110±0.026	0.095±0.021	0.235±0.054	0.577±0.030	0.773±0.054	1.000±0.178
154	1733	c	Terpinyl acetate	1.000±0.223	0.008±0.003	0.017±0.002	0.027±0.006	0.066±0.004	0.082±0.007	0.108±0.020
68	1771	b	*trans*-Linalool oxide, pyran	0.236±0.037	0.214±0.058	0.216±0.062	0.373±0.017	0.678±0.085	0.713±0.068	1.000±0.072
68	1787	b	*cis*-Linalool oxide, pyran	0.291±0.024	0.260±0.075	0.390±0.081	0.381±0.016	0.759±0.060	0.733±0.065	1.000±0.072
69	1824	b	Nerol (2010)*	0.039±0.007	0.042±0.002	0.014±0.003	0.193±0.037	0.548±0.059	0.641±0.053	1.000±0.218
69	1867	a	Geraniol (2010)*	0.041±0.013	0.044±0.005	0.013±0.003	0.159±0.049	0.528±0.064	0.656±0.054	1.000±0.283
71	1984	b	6,7-Dihydro-7-hydroxylinalool	0.034±0.001	0.033±0.006	0.059±0.017	0.176±0.045	0.616±0.054	0.584±0.065	1.000±0.187
81	2137	a	Terpin	0.131±0.052	0.123±0.049	0.271±0.085	0.314±0.050	0.744±0.114	0.630±0.100	1.000±0.064
81	2216	a	Terpin Hydrate	0.101±0.040	0.094±0.040	0.383±0.142	0.293±0.069	0.649±0.106	0.560±0.152	1.000±0.161
			C_{13} norisoprenoids							
142	1781	c	1,2-dihydro-1,1,6-trimethyl-naphthalene	0.034±0.006	0.056±0.007	0.329±0.042	0.593±0.096	1.000±0.116	0.988±0.166	0.812±0.124
119	1581	b	Vitispirane A + B	0.058±0.004	0.150±0.044	0.201±0.024	0.550±0.117	0.922±0.177	1.000±0.145	0.973±0.167
121	1874	c	β-Damascenone	0.130±0.020	0.172±0.025	0.299±0.025	0.802±0.047	0.956±0.117	0.947±0.210	1.000±0.137
163	1987	b	Actinidiol I	0.028±0.003	0.054±0.011	0.098±0.017	0.481±0.063	0.852±0.103	0.873±0.124	1.000±0.224
163	2001	b	Actinidiol II	0.030±0.004	0.058±0.012	0.113±0.020	0.526±0.063	0.879±0.104	0.919±0.124	1.000±0.222
157	2067	a	1,2-dihydro-1,5,8-trimethyl-naphthalene	0.027±0.004	0.048±0.006	0.088±0.010	0.490±0.059	0.854±0.040	1.000±0.038	0.959±0.091
132	2301	a	1-(2,3,6-trimethylphenyl)-2-Butanone	0.077±0.011	0.143±0.017	0.376±0.042	0.626±0.116	0.958±0.100	0.945±0.045	1.000±0.151
173	2368	a	1-(2,3,6-trimethylphenyl)-3-Buten-2-one	0.089±0.045	0.149±0.018	0.590±0.047	0.699±0.065	0.988±0.085	1.000±0.186	0.911±0.061
			Benzenoids							
124	1904	c	Guaicol	0.738±0.037	0.671±0.127	1.000±0.226	0.779±0.283	0.971±0.180	0.511±0.070	0.855±0.052
108	1918	c	Benzyl Alcohol	0.479±0.040	0.455±0.033	1.000±0.119	0.874±0.060	0.867±0.141	0.665±0.142	0.992±0.142

156	1959	c	Phenylethyl Alcohol	0.196±0.025	0.216±0.023	0.463±0.033	0.619±0.145	0.851±0.143	0.718±0.123	1.000±0.174
150	2246	c	2-Methoxy-4-vinylphenol	0.390±0.062	0.272±0.072	0.400±0.042	0.561±0.076	0.778±0.115	0.587±0.050	1.000±0.185
154	2313	c	Syringol	0.267±0.043	0.266±0.087	0.650±0.140	0.585±0.171	0.813±0.099	0.654±0.110	1.000±0.073
194	2369	b	2,6-dimethoxy-4-(2-prepenyl)-phenol	0.093±0.000	0.133±0.020	0.440±0.109	0.611±0.092	0.914±0.078	0.958±0.098	1.000±0.139
152	2628	c	Vanillin	0.507±0.137	0.656±0.160	0.485±0.073	0.639±0.125	0.893±0.029	0.565±0.099	1.000±0.196
151	2659	b	Methyl vanillate	0.250±0.028	0.311±0.073	0.776±0.098	0.716±0.074	0.865±0.070	0.801±0.052	1.000±0.199
151	2701	c	Acetovanilone	0.459±0.031	0.479±0.078	1.000±0.128	0.670±0.059	0.854±0.059	0.758±0.043	0.925±0.160
137	2713	a	Methyl vanillyl ketone	0.143±0.054	0.223±0.077	0.506±0.092	0.827±0.155	0.941±0.095	0.663±0.121	1.000±0.116
137	2845	c	Gingerone	0.305±0.029	0.328±0.065	1.000±0.188	0.405±0.040	0.511±0.047	0.547±0.065	0.983±0.210
137	2891	c	Homovanillyl alcohol	0.035±0.002	0.038±0.005	0.050±0.017	0.040±0.009	0.229±0.040	0.309±0.096	1.000±0.292
182	2987	c	Syringaaldehyde	0.237±0.052	0.315±0.094	0.265±0.040	0.599±0.144	0.736±0.201	0.712±0.120	1.000±0.076
137	3025	c	Homovanilic acid	0.369±0.152	0.320±0.128	0.357±0.090	0.620±0.036	0.780±0.124	0.562±0.044	1.000±0.175

[1]Quant mass was the automatically assigned unique mass by Leco deconvolution software.

[2]Retention index on DB-Wax column

[3]Identification was performed by library search (a) with literature retention index (b) and standard verification (c)

[4]Five monoterpenes odorants with an * sign were only detected in 2010 season

[5]The values reported were normalized to the maximum level during ripening period.

The level of 4 field replicates was acquired by dividing peak area of analyte to peak area of internal standard.

[6]Internal standards: 2-octanol for monoterpenes, 3-ethyl-3-dodecanol for C_{13} norisoprenoids, 2-secbutylphenol for benzenoids

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Chapter 6

Evaluating Effect of Timing and Frequency of Hedging On Acid-hydrolyzed Aglycones in *Vitis vinifera* L. cv. Riesling

Introduction

Studies have shown that plants emit a blend of volatile compounds (*1, 2*), particularly the green leaf volatiles - a resemblance of the cut grass aromas (*3, 4*) - when they are exposed to biotic and abiotic stresses. A biotic stress refers to attacks induced by living organisms such as pests while an abiotic stress refers to a non-living induced stress from the environment such as drought stress. The emission of an array of volatiles in response to attacks or stresses has been known as a defense mechanism in plant, which can be either a direct or indirect defenses (*1*). A direct defense is a direct way of deterring herbivores by emission of various volatiles such as terpenes that are toxic to the attackers (*5*). In the term of indirect defense, volatiles are used to attract carnivores that are predator to the herbivores (*6, 7*). Additionally, emission of volatiles also acts as an alarming signal to the neighboring plants (*8*). This tritrophic interaction among herbivore, carnivore and plant has been reported to occur throughout plant kingdom (*6*). In viticulture, grape vines are exposed to a physical wounding upon hedging which is one of the common viticultural practices. Hedging can be done by trimming off the shoot tips from the side or the top of the vines to obtain desirable canopy architecture. The frequency of this practice varies depending on the desirable canopy structure. Although hedging is a common cultural practice, no study has ever examined if this

treatment can cause sufficient abiotic stresses on grape vine thus altering the composition of volatiles produced in grapes. In grapevine, studies have reported the mechanism of detoxification by glycosylation (*9*). For instance, guaicol, a smoky aroma compound generated from bush fire, has been reported to be glycosylated in grapes and later to be released during winemaking (*9*). Presumably, wounding induced by hedging could induce more production of volatiles in grapes that are further glycosylated. To find out if hedging treatment can cause sufficient abiotic stress to stimulate grapevine in activating its defense mechanism and producing more glycosides, we measured glycosides in *Vitis vinifera* Riesling grapes collected from vines that had been subjected for hedging treatment at various timings and frequencies during fruit maturation period for 2009 and 2010. At harvest (56 dpv), grapes of all treatments were harvested and made into wine aiming for future wine aroma analysis and sensory evaluation.

Materials and method

Chemicals

All chemicals and aroma standards were purchased from Fischer Scientific (Pittsburgh, PA) and Sigma Aldrich (Allentown, PA) respectively. Solid phase extraction (SPE) cartridges packed with 200 mg LiChrolut EN sorbent (Merck, Darmstadt, Germany) were purchased from VWR International (West Chester, PA).

Experimental vineyard specification

In 2007, Riesling (clone 110) was cultivated on rootstock 3309 planted with a spacing of 2.75 m between rows and 1.10 m between vines at Cornell University experimental vineyard in the Cayuga Lake AVA. The experimental vines were pruned to 3 canes per vine with 12 nodes per cane and trained to a Pendelbogen training system. Of the 60 panels (3 rows of 20 panels per row) on the site, 24 panels were randomly selected for 6 treatments X 4 replicates. We randomly assigned 4 panels representing 4 biological replicates throughout the 3 rows. For the second season, panels for treatments and their replicates was randomly re-assigned.

Hedging treatment

Hedging was performed using a hedge trimmer model C – 016 from Echo Inc. (Lake Zurich, IL). This treatment was done at various times and frequencies during fruit maturation. Its treatment detail followed by sample collection for both seasons can be found in Table 1. Per replicate, about 300 g of berries were collected from the middle 3 vines. The sampling protocol was set at +2, +7, and +21 days post hedging treatment. The 6 treatments for 2009 were: 1) Control (no hedging), 2) VO (hedged at 0 days post-veraison), 3) V21 (21 days post-veraison), 4) V42 (42 days post-veraison), 5) V49 (49 days post-veraison), and 6) 2X (2 times hedging at 0 and 21 days post-veraison). For 2010, the treatments were: 1) Control, 2) V0, 3) V42, 4) Pre-V (-14 days post-veraison), 5) 2X (at 0 and 21 days post-veraison), and 6) 3X (at -14, 21 and 42

days post-veraison). For control, the grown shoots were manually tied up onto sticks to obtain an upright position thus preventing shoots from falling to the sides and causing undesirable shading.

Table 1 – Outline of sampling and hedging protocols for 2009 and 2010 seasons

[1]Dates and days post-veraison **2009**									
8/25	8/27	9/1	9/15	9/17	9/22	10/6	10/8	10/13	10/20
0	**+2**	**+7**	**+21**	**+23**	**+28**	**+42**	**+44**	**+49**	**harvest**
√	2X	2X	2X + √	2X	2X	2X			2X
√	V0	V0	V0			V0			V0
			√	V21	V21	V21			V21
						√	V42	V42	V42
								√	V49
	C	C	C	C	C	C	C	C	C

[1]Dates and days post-veraison **2010**												
7/28	7/30	8/4	8/11	8/13	8/18	9/1	9/3	9/8	9/22	9/24	9/29	10/13
-14	**-12**	**-7**	**0**	**+2**	**+7**	**+21**	**+23**	**+28**	**+42**	**+44**	**+49**	**harvest**
			√	V0	V0	V0			V0			V0
									√	V42	V42	V42
			√	2X	2X	2X+ √	2X	2X	2X			2X
√	3X	3X			3X	√	3X	3X	3X + √	3X	3X	3X
√	Pre-V	Pre-V			Pre-V			pre-V				Pre-V
	C	C		C	C	C	C	C	C	C	C	C

Treatment codes: C=no hedging control, **V0**= hedged at 0 days post veraison, **V21** = at 21 days post-veraison, **V42** = at 42 days post-veraison, **V49** = at 49 days post-veraison, **2X** = 2 times hedging at 0 and 21 days post-veraison, **3X** = 3 times at -14, 21, and 42 days post-veraison, **Pre-V**= at -14 days post-veraison. **Harvest** = +56 days post-veraison. [1] The treatment code indicated the time for sample collection while symbol √ indicated the time for hedging. Veraison for 2009 was on Aug 25th and 2010 was on Aug 11th.

Weather data collection

Weather data of daily temperature and precipitation from April 1st to Oct 31st can be accessed at: http://newa.cornell.edu/index.php?page=all-weather-data. The cumulative growing degree days (GDD) was measured based on 10 °C.

Sample preparation

Once berries were collected, they were kept at -20 °C until the glycoside analysis. For the analysis, we used a Waring blender (model no. 5011, Torrington, CT) to blend 200 g of thawed berries, then loaded into 85 mL-NALGENE polycarbonate centrifuge tubes (VWR International, West Chester, PA), and centrifuged for 30 min at 10,000 rpm and 5 °C (5810 R Centrifuge, VWR International). After centrifuging, the supernatant (juice) was filtered through a No. 41 Whatman filter paper. The supernatant was then subjected to glycoside analysis.

Glycoside analysis

Previously optimized SPE method (*10*) for glycosides extraction were adopted in this study using a Varian 24-cartridge Positive Pressure Manifold (Palo Alto, CA). For this procedure, glycosides were first isolated and extracted using solid phase extraction (SPE). Next, the glycoside extract was dried down completely, reconstituted with buffer, and then hydrolyzed at 100 °C for 1 hr to liberate the aromas from their bound forms. Finally, the liberated aromas were

isolated and extracted using the second SPE. The details of this procedure can be cited in Chapter 5.

Enhanced point quadrat analysis (EPQA)

The EPQA measurement was adopted from Mayer et al. 2008 (*11*) and was applied to assess sunlight interception and canopy biomass distribution at the fruiting zone. In 2009, this analysis was carried out both pre- and post-hedging. In 2010, the measurement was conducted at pre-veraison prior to hedging and post-veraison at 2 weeks before harvest.

Fermentation condition

At harvest or 56 days post-veraison (Oct 20th, 2009 and Oct 13th, 2010), grape clusters of the middle 3 vines were manually picked and transferred to 3 °C storage room prior to de-stemming. The 4 replicates per treatment were combined, de-stemmed, crushed and pressed. The pressed juice (*ca.* 50% w/w of the crushed grapes) was clarified by settling for 24 hours at 20 °C. Per treatment, the clarified juice was vinified in duplicate 3.785 L carboy with 0.25 g/L of Uvaferm CEG yeast at 20 °C. Diammonium phosphate (DAP) (0.75 g/L) and Fermaid K (0.25 g/L) were added when the total soluble solids had been reduced by approximately 30%. Once fermentation had reached to a complete dryness (<1% concentration of residual sugar, verified by clinic test), wine was racked off from yeast lees, added SO_2 (90 mg/L), and cold stabilized for 3 months at 3 °C. After cold stabilization, wine was racked off once more from

the fine lees, then added SO_2 (40 mg/L) and bottled in a 375 mL amber wine bottle topped with natural cork.

GC-TOF-MS

Sample analysis was performed with DB-Wax column (60 m × 0.25 mm × 0.50 µm, Varian, Walnut Creek, CA), connected to a VF-17 ms (1 m × 0.1 mm × 0.2 µm, Varian). Although the system was set up for GCxGC analyses, the GC×GC modulator was turned off during analysis resulting in 1-D GC−TOF-MS. The sample was injected splitless with an injector temperature set to 250 ºC. The purge was opened after 2 min. Helium was used as a carrier gas at a flow rate of 1 mL/min. The temperature program was as follows: initial hold for 1 min at 55 ºC, followed by 3 ºC/min to 240 ºC, 60 min hold. The secondary column and modulator temperature offset was +15 ºC. The MS transfer line temperature was 260 ºC. The TOF-MS was operated in EI mode with an ionization energy of 70 eV. The electron multiplier was set to 1700 V. MS data from *m*/*z* 20−400 were stored at an effective sampling rate of 5 Hz.

Data processing

Data processing was carried out by Leco ChromaTOF software where unique ion was used for peak integration. Peak identification was performed by NIST library search in combination with literature retention index and/or authentic standard verification. For semi-quantitative comparison, peak ratio, generated by analyte peak area divided by internal standard peak area, was normalized

to the maximum value. The internal standard mixture covering 4-hydroxy-4-methyl-2-pentanone, pentanoic acid, 2-octanol, 3-ethyl-3-dodecanol, and 2-*sec*-butyl-phenol was used to quantify ketones, fatty acids, monoterpenes, C_{13} norisoprenoids, and benzenoids respectively.

Statistical Analysis

Statistical analysis was performed by JMP version 8 (SAS Institute, Cary, NC) using ANOVA; Student's *t* test, Tukey HSD comparison.

Results and Discussion

The climate conditions of the 2 seasons are very different – warmer and wetter in 2010 than in 2009. The details of total growing degree days and precipitation is available in Chapter 5.

Basic juice parameters

At harvest (56 days post-veraison), total soluble solids, pH, and titratable acids were measured in all treatments. In both seasons, the results showed no difference between hedged and non-hedged vines except for the 2009 - V56 sample where the soluble solid was slightly higher and the pH was lower (Table 2). Since hedging did not alter the amount of clusters per vine and the intensity of light interception in the fruiting zone, progress of fruit maturity of the treated vines were neither be advanced nor delayed. Thus, it was not surprising that there was no difference on ripening process between hedged

and non-hedged vines. Comparing the 2 seasons, the levels of total soluble solid and pH were comparable having an average 20.8 °Brix in 2009 and average 21.2 °Brix in 2010 and pH 3.05 (2009) to 3.03 (2010). The titratable acids were significantly higher in 2009 (10.8 g/L) than in 2010 (7.3 g/L).

Table 2 – Values of basic juice parameters of different treatments

	Total soluble solid (°Brix)	pH	Titratable acids (g/L)
2009	*	*	ns
Control	21.1±0.2 [ab]	3.06 [a]	10.7±0.3
2X	20.5±0.4 [b]	3.05 [ab]	10.6±0.7
VO	20.3±0.4 [b]	3.08 [a]	11.3±0.5
V21	20.6±0.1 [b]	3.05 [ab]	10.8±0.5
V42	21.0±0.1 [ab]	3.05 [a]	10.5±0.4
V56	21.8±0.1 [a]	3.00 [b]	10.9±0.1
2010	ns	ns	ns
Control	21.3±0.2	3.04	7.2±0.1
Pre-V	21.2±0.3	3.05	7.0±0.1
V0	21.3±0.1	3.04	7.5±0.2
V42	21.3±0.2	3.01	7.6±0.3
2X	20.8±0.3	3.03	7.5±0.1
3X	21.4±0.3	3.03	7.1±0.1

* indicated statistical significant difference (Anova, $p<0.05$) while ns indicated no significant difference $p>0.05$.

EPQA analysis

EPQA analysis was measured before and after hedging in 2009 and at pre-veraison prior to hedging and post-veraison (2 weeks before harvest) in 2010. As shown in Figure 1 for 2010 data, the levels of cluster exposure in treated vines were *ca.* ±10% to the untreated vines from deeply shaded fruit to highly exposed fruit zones for both values taken at pre- and post-veraison. In 2009, the levels of cluster exposure in all treated vines were *ca.* ±10% to the

untreated control (graph not shown). This small variation suggested that the hedging treatment did not significantly alter the canopy structure of the vines.

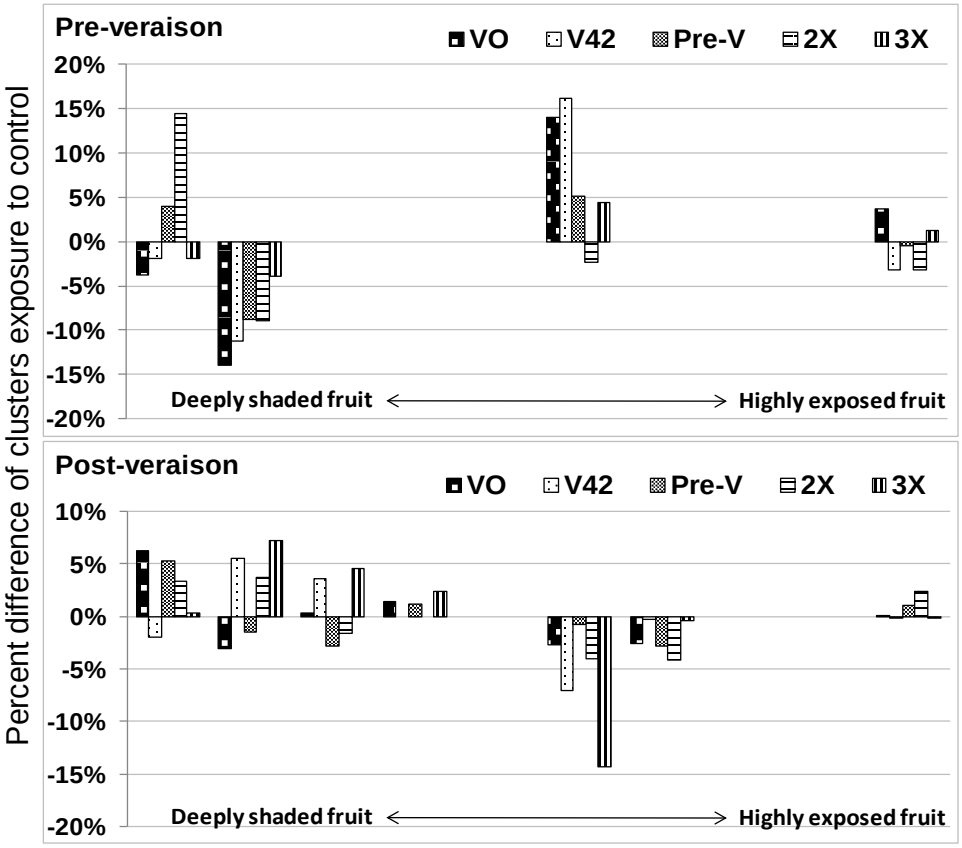

Figure 1 – Cluster exposure maps showing percent difference to control (no hedged vines) measured at pre-veraison and at harvest in 2010. **Treatment codes:** V0= hedged at 0 days post veraison, V42 = at 42 days post-veraison, 2X = 2 times hedging at 0 and 21 days post-veraison, 3X = 3 times at -14, 21, and 42 days post-veraison, and Pre-V= at -14 days post-veraison. EPQA for 2009 was measured immediately before and after hedging. The percent differences to control for all treatments were within ±10%.

Impact of hedging on acid-hydrolyzed aglycone composition

Studies evaluating impacts of viticultural practices on wine aromas are very challenging because these practices integrate many growing parameters that are strongly dependent among each other. For instance, various pruning and training systems produce different levels of crop yield, canopy microclimate, vine's vigor, and light interception. Leaf removal will alter the canopy air flow,

canopy microclimate, and canopy light interception. It is very challenging to make sure that the specific cultural practice changes only the targeted growing parameters without altering the others. In hedging study, the treatment of trimming of shoot tips was intentionally carried out to trim from the top of the vine, not from the side panels. This is to avoid alteration of light interception into the side panels of canopy especially into the fruiting zone. To evaluate acid-hydrolyzed aglycone composition in grape juice for the 2009 and 2010 seasons, we performed hedging treatment at different timings and intensities. Although small differences were noted among treatments, hedging did not significantly (Anova, $p>0.05$) alter monoterpene, C_{13} norisoprenoid, and benzenoid aglycone compositions as shown in Figure 2, 3, and 4 respectively. We noted that the biological variation of the 4 replicates is larger than the degree of impact induced by hedging treatments at different timings and intensities. Although no study has ever examined the impact of hedging on bound volatiles in grape juice, many studies have evaluated impact of different cultural practices on bound volatiles such as leaf removal (*12-15*), nitrogen fertilization (*16, 17*), and water deficiency (*18, 19*). Results of these studies showed both increase and decrease of bound volatiles. Basal leaves removal showed no impact on the sum of free and bound monoterpenes in Riesling (*15*) while fruiting zone leaves removal showed higher glycoside levels by means of phenol-free G-G level (*13*). Compared to the shaded ones, sun-exposed clusters were associated with higher TDN and Vitispirane released by acid-hydrolysis in South African Riesling (*20*) and the timing of the exposure

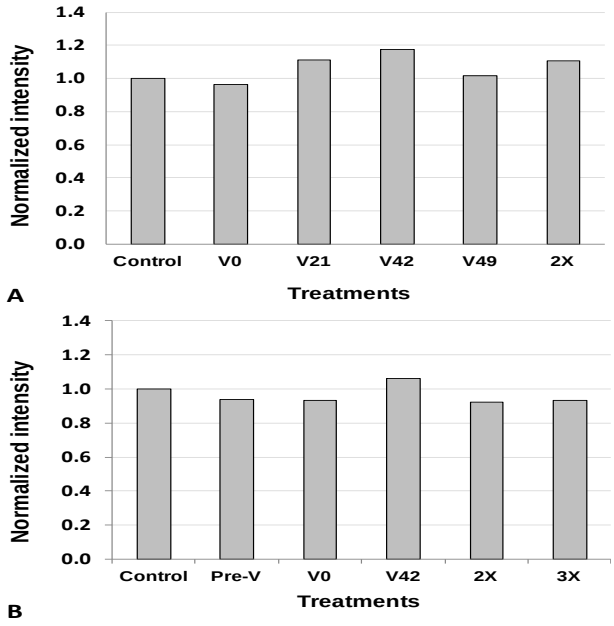

Figure 2 – In 2009 (A) and 2010 (B) monoterpene aglycones, no significant difference was noted among treatments (p>0.05, Anova). The response was generated by normalizing the ratio of each monoterpenes per treatment to control and then averaging the normalized monoterpenes per treatment. Supplementary Table 1 and 2 provided the list of monoterpenes for 2009 and 2010 respectively.

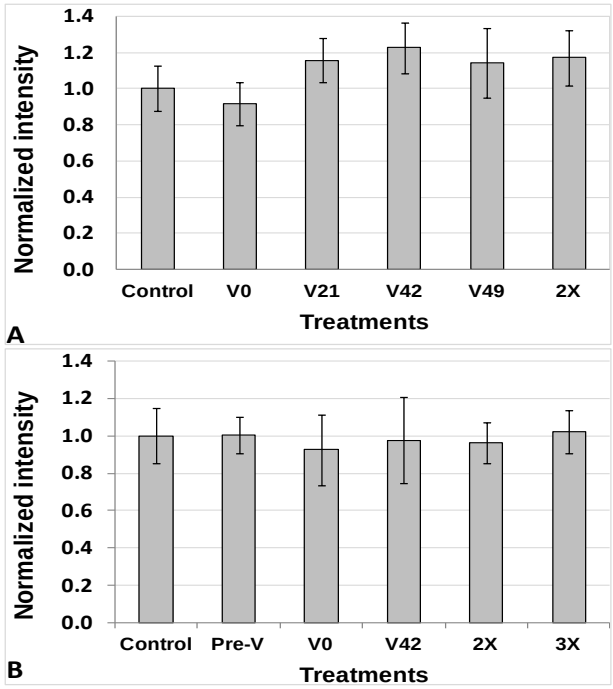

Figure 3 – In 2009 (A) and 2010 (B) C_{13} norisoprenoid aglycones, no significant difference was noted among treatments (p>0.05, Anova). The response was generated by normalizing the ratio of each C_{13} norisoprenoid per treatment to control and then averaging the normalized C_{13} norisoprenoids per treatment. Supplementary Table 1 and 2 provided the list of monoterpenes for 2009 and 2010 respectively.

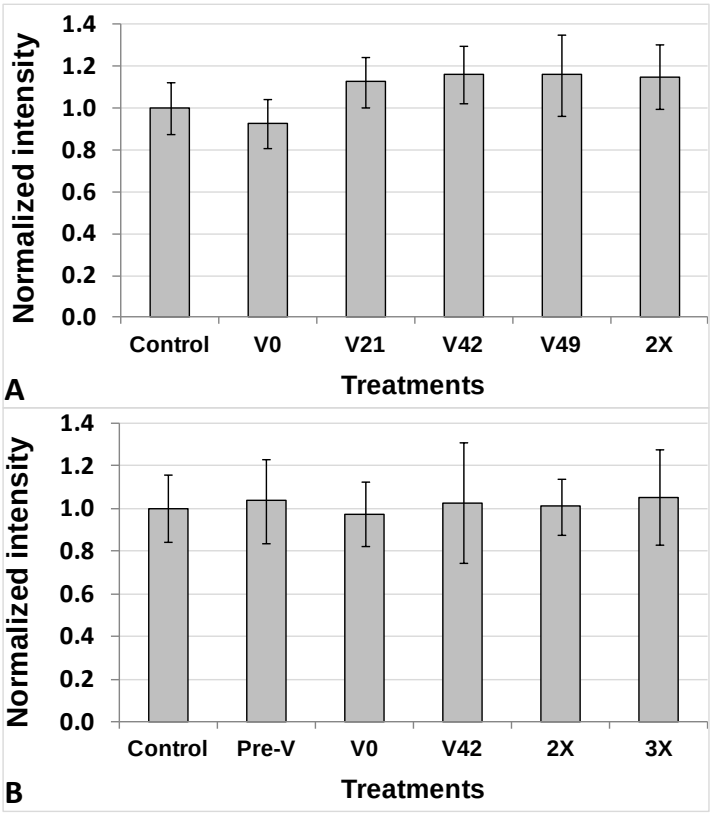

Figure 4 – In 2009 (A) and 2010 (B) benzenoid aglycones, no significant difference was noted among treatments (p>0.05, Anova). The response was generated by normalizing the ratio of each benzenoid per treatment to control and then averaging the normalized benzenoids per treatment. Supplementary Table 1 and 2 provided the list of monoterpenes for 2009 and 2010 respectively.

was reported to be pivotal showing highest at 33 days post-veraison (*12*). One critical point to note on the results of these studies is that they are using different analytical techniques to measure the bound volatiles. Thus, a direct comparison of the results could be very challenging. Like hedging, leaf removal practice is regularly performed to attain desirable canopy structure. However, unlike hedging which induces physical wounding, leaf removal alters the light interception into the canopy. Although our result did not show significant difference in the three aroma classes, we noted significant differences (p<0.05, Anova) on Vitispirane A + B, 1,2-dihydro-1,5,8-trimethyl-

napthalene, 1-(2,3,6-trimethylphenyl)-2-butanone, and syringol in 2009 season and guaicol in 2010 season as shown in supplementary Table 1 and 2 respectively. The differences of compounds in 2009 are minor and are not consistently expressed again in the following season. The sampling points prior to harvest did not show significant differences on these compounds. Thus, it cannot be concluded that these differences are induced by hedging treatment. In 2010, the difference of guaicol concentration among treatment is highly significant, $p<0.0001$. However, the difference was not noted on earlier time points. It was not clear why guaicol increased significantly at the last time point where an intense level of *B.cinerea* infection was noted. Nitrogen fertilization showed no impact on monoterpene profile on one study (*16*) while significant difference was noted in aged Riesling on the other study (*17*). Water deficit was reported to increase phenol-free G-G level (*18*) and bound monoterpenols and nor-isoprenoids (*21*).

Conclusions

Conclusively, the applied hedging treatments at different intensities and timings did not significantly alter acid-hydrolyzed aglycone composition although small differences were noted among treatments.

Supplementary Table 1 – Values of acid hydrolyzed aglycones of monoterpenes, C_{13} norisoprenoids, and benzenoids by hedging treatments for 2009 season

No.	Quant Mass[1]	RI[2]	ID[3]	Analytes	Control[4,5]	2X	V0	V21	V42	V56	Anova $p<0.05$
				Monoterpenes							
1	139	1011	a	2-ethenyltetrahydro-2,6,6-trimethyl-2H-pyran	1.00±0.20	1.19±0.20	0.98±0.22	1.17±0.31	1.19±0.10	1.10±0.16	ns
2	68	1137	b	Limonene	1.00±0.32	1.08±0.10	0.89±0.19	1.08±0.30	1.21±0.11	0.88±0.21	ns
3	111	1191	c	Eucalyptol	1.00±0.15	1.22±0.24	1.02±0.31	1.18±0.31	1.23±0.15	0.99±0.23	ns
4	93	1212	b	*trans*-Ocimene	1.00±0.33	1.02±0.11	0.91±0.26	1.00±0.39	1.14±0.04	0.77±0.23	ns
5	139	1227	a	tetrahydro-2,2-dimethyl-5-(1-methyl-1-propenyl)-furan	1.00±0.22	1.15±0.28	1.07±0.34	1.13±0.40	1.14±0.07	0.91±0.23	ns
6	93	1233	b	Gamma terpinene	1.00±0.25	1.08±0.10	0.96±0.23	1.05±0.30	1.23±0.11	0.84±0.29	ns
7	119	1261	b	*p*-Cymene	1.00±0.14	1.07±0.04	0.94±0.12	1.10±0.20	1.22±0.20	0.86±0.28	ns
8	93	1276	b	Terpinolene	1.00±0.28	1.12±0.09	0.96±0.19	1.09±0.31	1.21±0.04	0.85±0.33	ns
9	94	1444	b	*trans*-linalool oxide furan	1.00±0.14	1.08±0.16	0.90±0.20	1.11±0.15	1.14±0.07	1.10±0.19	ns
10	59	1474	b	*cis*-linalool oxide furan	1.00±0.15	1.15±0.18	0.89±0.21	1.16±0.17	1.20±0.12	1.14±0.19	ns
11	85	1476	b	Nerol oxide	1.00±0.16	1.03±0.07	0.92±0.21	1.13±0.21	1.15±0.06	1.04±0.19	ns
12	93	1536	c	Linalyl acetate	1.00±0.19	1.10±0.11	0.98±0.11	1.07±0.21	1.09±0.09	1.01±0.10	ns
13	81	1580	b	1-Terpinenol	1.00±0.08	1.12±0.15	1.02±0.24	1.14±0.25	1.20±0.15	1.09±0.18	ns
14	80	1602	b	Myrcenol	1.00±0.19	1.06±0.13	1.00±0.21	1.07±0.27	1.11±0.02	1.00±0.15	ns
15	82	1603	b	Hotrienol	1.00±0.18	0.88±0.10	0.93±0.06	0.99±0.24	1.01±0.15	0.89±0.16	ns
16	111	1616	b	4-Terpineol	1.00±0.13	1.09±0.14	1.02±0.21	1.10±0.28	1.15±0.05	1.05±0.15	ns
17	93	1638	b	*cis*-α-Terpineol	1.00±0.14	1.12±0.12	1.04±0.24	1.12±0.29	1.15±0.05	1.07±0.16	ns
18	93	1645	b	*cis*-Ocimenol	1.00±0.19	1.10±0.22	1.05±0.27	1.09±0.35	1.12±0.03	1.06±0.18	ns
19	93	1672	b	*trans* Ocimenol	1.00±0.19	1.06±0.20	1.05±0.24	1.06±0.31	1.10±0.03	1.03±0.16	ns
20	59	1709	c	α-Terpineol	1.00±0.19	1.34±0.22	0.93±0.26	1.31±0.31	1.31±0.15	1.19±0.21	ns

21	154	1711	c	Terpinyl acetate	1.00±0.13	1.08±0.07	1.00±0.25	1.15±0.31	1.24±0.10	1.12±0.20	ns
22	68	1748	b	*trans*-linalool oxide, pyran	1.00±0.13	1.15±0.12	0.91±0.18	1.13±0.18	1.18±0.24	1.10±0.19	ns
23	68	1767	b	*cis*-linalool oxide, pyran	1.00±0.10	1.18±0.13	0.94±0.18	1.13±0.17	1.18±0.19	1.17±0.18	ns
24	71	1969	b	6,7-Dihydro-7-hydroxylinalool	1.00±0.12	0.94±0.11	0.88±0.22	0.96±0.29	1.06±0.14	0.95±0.17	ns
25	81	2116	a	Terpin	1.00±0.07	1.28±0.22	0.92±0.31	1.25±0.36	1.36±0.29	1.16±0.22	ns
26	81	2195	a	Terpin Hydrate	1.00±0.16	1.10±0.23	0.97±0.38	1.13±0.38	1.24±0.29	1.00±0.20	ns
				C13 norisopreniods							
27	119	1553	b	Vitispirane A + B	1.00±0.19	1.17±0.11	0.90±0.08	1.25±0.17	1.30±0.10	1.18±0.21	*
28	142	1781	c	1,2-dihydro-1,1,6-trimethyl- naphthalene	1.00±0.20	1.20±0.17	0.93±0.18	1.24±0.13	1.33±0.12	1.20±0.28	ns
29	121	1851	c	β-Damascenone	1.00±0.09	1.04±0.20	0.89±0.09	1.02±0.13	1.06±0.12	1.00±0.12	ns
30	163	1962	b	Actinidiol I	1.00±0.06	1.18±0.20	0.91±0.06	1.09±0.14	1.20±0.19	1.14±0.13	ns
31	163	1976	b	Actinidiol II	1.00±0.06	1.17±0.21	0.90±0.06	1.07±0.14	1.21±0.20	1.15±0.14	ns
32	157	2043	a	1,2-dihydro-1,5,8-trimethyl-naphthalene	1.00±0.16	1.19±0.09	0.92±0.13	1.20±0.12	1.28±0.12	1.17±0.21	*
33	132	2280	a	1-(2,3,6-trimethylphenyl)-2-butanone I	1.00±0.18	1.27±0.15	0.96±0.18	1.28±0.10	1.32±0.08	1.25±0.24	*
34	173	2349	a	1-(2,3,6-trimethylphenyl)-2-butanone II	1.00±0.05	1.14±0.10	0.94±0.17	1.10±0.04	1.11±0.17	1.05±0.21	ns
				Benzenoids							
35	124	1879	c	Guaiacol	1.00±0.14	1.17±0.15	0.89±0.14	1.09±0.23	1.14±0.30	1.11±0.10	ns
36	108	1893	c	Benzyl Alcohol	1.00±0.07	0.99±0.14	0.90±0.05	0.99±0.06	1.02±0.09	0.93±0.12	ns
37	156	1933	c	Phenylethyl Alcohol	1.00±0.05	1.13±0.15	0.90±0.10	1.13±0.10	1.19±0.18	1.00±0.14	ns
38	150	2224	c	2-Methoxy-4-vinylphenol	1.00±0.09	1.16±0.21	0.93±0.17	1.19±0.20	1.33±0.24	1.33±0.27	ns
39	154	2294	c	Syringol	1.00±0.18	1.57±0.43	0.86±0.14	1.29±0.25	1.45±0.45	1.38±0.24	*
40	154	2305	b	3,4-dimethoxy-phenol	1.00±0.11	1.12±0.17	1.06±0.15	1.02±0.09	1.12±0.15	1.24±0.20	ns
41	151	2609	c	Vanillin	1.00±0.10	1.23±0.23	1.01±0.11	1.22±0.32	1.18±0.10	1.23±0.16	ns

42	151	2641	b	Methyl vanillate	1.00±0.05	1.08±0.15	0.96±0.08	1.07±0.08	1.15±0.11	1.14±0.13	ns
43	151	2682	c	Acetovanilone	1.00±0.05	1.09±0.10	0.96±0.14	1.08±0.06	1.14±0.17	1.15±0.16	ns
44	137	2697	a	Methyl vanillyl ketone	1.00±0.18	0.97±0.14	0.96±0.10	1.01±0.11	1.04±0.22	1.01±0.11	ns
45	137	2831	c	Gingerone	1.00±0.14	1.19±0.10	0.88±0.24	1.21±0.14	1.21±0.28	1.20±0.23	ns
46	137	2874	c	Homovanillyl alcohol	1.00±0.35	1.20±0.37	0.99±0.29	1.37±0.43	1.14±0.26	1.21±0.28	ns
47	182	2971	c	Syringaldehyde	1.00±0.19	1.21±0.29	0.83±0.20	1.26±0.39	1.09±0.17	1.13±0.17	ns
48	137	3008	c	Homovanilic acid	1.00±0.25	0.99±0.21	0.83±0.20	0.84±0.19	1.05±0.20	1.15±0.06	ns

[1]Quant mass was the automatically assigned unique mass by Leco deconvolution software.
[2]Retention index on DB-Wax column.
[3]Identification was performed by library search (a) with literature retention index (b) and standard verification (c).
[4]The values reported were normalized to the control level.
[5]Internal standards: 2-octanol for monoterpenes, 3-ethyl-3-dodecanol for C13 norisoprenoids, 2-secbutylphenol for benzenoids.
ns and * indicated no significant difference and significant difference at $p<0.05$, Anova

Supplementary Table 2 – Values of acid hydrolyzed aglycones of monoterpenes, C_{13} norisoprenoids, and benzenoids by hedging treatments for 2010 season

Quant Mass[1]	RI[2]	ID[3]	Analytes	Control[4,5]	Pre-V	V0	V42	2X	3X	ANOVA $p<0.05$
			Monoterpenes							
139	1132	b	2-ethenyltetrahydro-2,6,6-trimethyl-2H-pyran	1.00±0.20	0.94±0.21	0.94±0.18	1.02±0.31	0.86±0.15	0.88±0.17	ns
93	1176	b	α-Myrcene	1.00±0.38	0.98±0.31	1.09±0.28	1.28±0.38	1.08±0.08	1.00±0.29	ns
68	1226	b	Limonene	1.00±0.35	0.99±0.28	1.11±0.23	1.35±0.42	1.12±0.10	1.00±0.23	ns
111	1243	c	Eucalyptol	1.00±0.20	0.97±0.22	0.94±0.16	1.06±0.31	0.88±0.19	0.92±0.18	ns
93	1248	b	*trans*-Ocimene	1.00±0.32	1.03±0.31	1.10±0.20	1.32±0.44	1.04±0.08	1.02±0.32	ns
139	1265	b	Tetrahydro-2,2-dimethyl-5-(1-methyl-1-propenyl)-furan	1.00±0.24	0.98±0.23	0.89±0.28	1.11±0.41	0.87±0.23	0.89±0.15	ns
93	1267	b	*cis*-Ocimene	1.00±0.28	1.05±0.30	1.12±0.21	1.31±0.45	1.04±0.10	1.04±0.33	ns
93	1272	b	Gamma terpinene	1.00±0.37	0.95±0.20	0.98±0.14	1.31±0.43	1.08±0.14	0.96±0.24	ns
119	1299	b	*p*-Cymene	1.00±0.33	0.88±0.08	0.84±0.19	1.13±0.27	0.99±0.18	0.97±0.29	ns
93	1313	b	Terpinolene	1.00±0.34	1.00±0.26	1.06±0.18	1.28±0.44	1.03±0.12	0.99±0.26	ns
94	1474	b	*trans*-linalool oxide, furan	1.00±0.08	0.85±0.11	0.98±0.17	1.00±0.27	0.91±0.07	0.89±0.19	ns
59	1503	b	*cis*-linalool oxide, furan	1.00±0.08	0.99±0.13	1.01±0.12	1.04±0.19	0.98±0.03	1.02±0.19	ns
85	1503	b	Nerol oxide	1.00±0.10	0.99±0.16	0.98±0.05	1.05±0.25	1.00±0.06	1.00±0.21	ns
71	1555	c	Linalool	1.00±0.10	0.97±0.20	1.03±0.21	1.03±0.21	0.87±0.07	0.96±0.19	ns
93	1555	c	Linalyl acetate	1.00±0.16	0.96±0.27	0.89±0.19	0.99±0.19	0.88±0.05	0.98±0.29	ns
81	1605	b	1-Terpinenol	1.00±0.12	0.89±0.10	0.83±0.19	1.02±0.22	0.89±0.16	0.90±0.20	ns
59	1622	b	Myrcenol	1.00±0.09	1.06±0.23	0.98±0.18	1.07±0.35	0.88±0.13	0.98±0.19	ns
82	1625	b	Hotrienol	1.00±0.10	0.97±0.16	0.96±0.17	1.00±0.12	0.97±0.07	1.02±0.17	ns
111	1642	b	4-Terpineol	1.00±0.13	0.91±0.17	0.88±0.11	1.00±0.25	0.86±0.12	0.87±0.18	ns
71	1662	b	*cis*-α-Terpineol	1.00±0.10	0.88±0.12	0.88±0.18	0.99±0.26	0.88±0.13	0.86±0.19	ns

93	1664	b	*cis*-Ocimenol	1.00±0.14	0.91±0.20	0.87±0.18	0.98±0.37	0.81±0.15	0.85±0.18	ns
93	1691	b	*trans*-Ocimenol	1.00±0.13	0.95±0.22	0.91±0.20	1.06±0.43	0.80±0.13	0.89±0.18	ns
59	1733	c	α-Terpineol	1.00±0.18	1.03±0.18	0.93±0.14	1.05±0.09	0.92±0.08	1.02±0.13	ns
154	1733	c	Terpinyl acetate	1.00±0.20	0.92±0.15	0.83±0.10	1.04±0.21	0.86±0.15	0.76±0.22	ns
68	1771	b	*trans*-linalool oxide, pyran	1.00±0.08	0.77±0.10	0.79±0.13	0.82±0.20	0.74±0.05	0.74±0.15	ns
68	1788	b	*cis*-linalool oxide pyran	1.00±0.07	0.89±0.14	0.91±0.14	0.95±0.20	0.89±0.04	0.87±0.16	ns
69	1825	b	Nerol	1.00±0.11	0.90±0.27	0.77±0.10	0.99±0.15	0.83±0.01	0.92±0.24	ns
69	1868	b	Geraniol	1.00±0.17	0.95±0.30	0.81±0.12	0.78±0.31	0.85±0.03	0.96±0.26	ns
71	1985	b	6,7-Dihydro-7-hydroxylinalool	1.00±0.13	0.90±0.19	1.03±0.31	1.04±0.19	0.92±0.10	0.96±0.27	ns
81	2138	a	Terpin	1.00±0.16	0.89±0.12	0.89±0.10	0.83±0.44	0.94±0.15	0.95±0.20	ns
81	2216	a	Terpin Hydrate	1.00±0.16	0.79±0.09	0.72±0.13	1.09±0.25	0.87±0.17	0.85±0.25	ns
			C13 norisoprenoids							
119	1580	b	Vitispirane A + B	1.00±0.12	1.11±0.08	1.07±0.32	1.06±0.31	1.09±0.08	1.17±0.12	ns
157	1807	c	1,2-dihydro-1,1,6-trimethyl-naphthalene	1.00±0.15	1.15±0.10	1.01±0.39	1.11±0.42	1.04±0.16	1.14±0.13	ns
121	1875	c	β-Damascenone	1.00±0.14	0.91±0.19	0.91±0.05	0.97±0.12	1.08±0.15	1.03±0.15	ns
163	1987	b	Actinidiol I	1.00±0.23	0.96±0.07	0.95±0.15	0.94±0.16	0.96±0.11	1.03±0.13	ns
163	2002	b	Actinidiol II	1.00±0.23	0.97±0.08	0.94±0.15	0.94±0.15	0.97±0.10	1.06±0.10	ns
157	2068	a	1,2-dihydro-1,5,8-trimethyl-naphthalene	1.00±0.09	1.02±0.09	0.91±0.14	0.98±0.23	0.90±0.08	1.01±0.09	ns
132	2301	a	1-(2,3,6-trimethylphenyl)-2-butanone I	1.00±0.11	1.00±0.08	0.86±0.19	0.95±0.27	0.86±0.09	0.95±0.10	ns
157	2434	a	1-(2,3,6-trimethylphenyl)-2-butanone II	1.00±0.13	0.95±0.09	0.85±0.11	0.89±0.10	0.92±0.08	0.89±0.18	ns
157	2528	a	1-(2,3,6-trimethylphenyl)-2-butanone III	1.00±0.12	0.98±0.07	0.83±0.22	0.94±0.29	0.85±0.13	0.94±0.04	ns
			Benzenoids							
124	1905	c	Guaicol	1.00±0.12	1.73±0.16	1.73±0.18	1.92±0.15	1.64±0.19	2.06±0.18	<0.0001

108	1918	c	Benzyl Alcohol	1.00±0.10	0.95±0.11	0.88±0.15	1.00±0.09	0.93±0.12	0.91±0.04	ns
92	1955	c	Phenylethanol	1.00±0.12	1.10±0.12	0.95±0.10	1.00±0.20	1.06±0.12	0.97±0.10	ns
135	2246	c	2-Methoxy-4-vinylphenol	1.00±0.19	1.07±0.12	1.08±0.10	0.85±0.57	1.07±0.14	1.19±0.13	ns
154	2308	c	Syringol	1.00±0.07	1.07±0.09	1.12±0.10	0.93±0.42	1.15±0.11	1.20±0.15	ns
151	2628	c	Vanillin	1.00±0.21	0.89±0.25	0.76±0.20	0.80±0.11	0.85±0.09	0.78±0.14	ns
151	2659	b	Methyl vanillate	1.00±0.19	0.99±0.18	0.86±0.11	0.96±0.12	0.89±0.08	0.89±0.16	ns
151	2701	c	Acetovanilone	1.00±0.17	1.03±0.06	0.91±0.09	1.04±0.12	0.93±0.07	0.95±0.32	ns
137	2713	a	Methyl vanillyl ketone	1.00±0.10	1.20±0.24	0.95±0.07	1.30±0.29	1.15±0.14	1.15±0.57	ns
151	2860	c	Gingerone	1.00±0.02	0.82±0.31	0.85±0.14	1.07±0.42	1.09±0.21	1.00±0.41	ns
137	2891	c	Homovanillyl alcohol	1.00±0.29	0.82±0.28	0.93±0.28	0.97±0.67	0.80±0.20	0.83±0.44	ns
182	2987	c	Syringaldehyde	1.00±0.31	0.90±0.35	0.79±0.26	0.66±0.21	0.77±0.16	0.78±0.10	ns
137	3025	c	Homovanilic acid	1.00±0.18	0.89±0.28	0.87±0.19	0.85±0.27	0.81±0.06	0.99±0.20	ns

[1]Quant mass was the automatically assigned unique mass by Leco deconvolution software.
[2]Retention index on DB-Wax column.
[3]Identification was performed by library search (a) with literature retention index (b) and standard verification (c).
[4]The values reported were normalized to the control level.
[5]Internal standards: 2-octanol for monoterpenes, 3-ethyl-3-dodecanol for C13 norisoprenoids, 2-secbutylphenol for benzenoids
ns and * indicated no significant difference and significant difference at $p<0.05$, Anova

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