QUANTIFICATION OF 3-ISOBUTYL-2-HYDROXYPYRAZINE, A KEY INTERMEDIATE IN IBMP METABOLISM

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ABSTRACT

3-isobutyl-2-hydroxypyrazine (IBHP) is thought to be a key intermediate in both the biosynthesis and degradation of the herbaceous smelling 3-isobutyl-2-methoxypyrazine (IBMP), but quantitative methods for IBHP analysis are not widely reported and its behavior over the growing season is not well understood. A recent hypothesis suggested that IBHP and IBMP concentrations over the growing season were correlated, leading to the ability to estimate pre-veraison IBMP from harvest IBHP samples.

An improved method for quantification of IBHP was developed, using a $^{2}[H_2]$-IBHP standard, isolation by mixed-mode cation exchange SPE and silylation prior to GC-TOF-MS analysis. A limit of detection of 33 ng/L could be achieved for a 100 ml juice sample. This method was used to quantify IBHP during the 2010 growing season at sites in both the New York Finger Lakes and the California Central Valley regions. Free IBHP increased pre-veraison until a maximum at veraison, and both free and bound IBHP decreased until harvest. In New York Cabernet franc, the concentration of IBHP increased to over 280 pg/berry and in California Merlot IBHP peaked to over 470 pg/berry. Peak concentrations of free IBHP correlated with peak concentrations of IBMP, with Merlot accumulating both the most IBMP and free IBHP. The decline of IBHP occurred at least 2 weeks after typical IBMP synthesis and degradation, though different varietals and clones have differing degradation patterns. Acid-labile IBHP in Cabernet franc degraded following veraison levels nearing 950 pg/berry, to levels statistically indistinguishable from free IBHP near harvest. The ability of several yeast strains to methylate IBHP into IBMP was examined, and it was found that the yeast strains studied did not synthesize IBMP under the fermentation conditions.

BIOGRAPHICAL SKETCH

Sarah Anne Harris was born and raised in the town of Los Alamos, New Mexico, where she quickly discovered her love of cooking was based on a love of science. She attended Cornell University, where she spent two years studying houseflies for her undergraduate research project resulting in the honors thesis *Frequencies of* Vssc *and* CYP6D1 *Alleles in House Flies from Florida, North Carolina, New York and Kansas,* and graduated in 2010 with a B.S. in Biology with High Honors. Once realizing she could combine two of her favorite interests, wine and chemistry, she continued her graduate career at Cornell University working under Dr. Gavin Sacks and studied wine flavor chemistry. She enjoys cooking, canning, and a good glass of wine, and plans to continue on to her goal of becoming a winemaker.

To the family I was given; and to Big Al, C-$ and Wif, the family I've chosen.

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CHAPTER 1

BACKGROUND

1.1 The Methoxypyrazines

3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-3-methoxypyrazine (IPMP) and *sec*-butyl-2-methoxypyrazine (*s*BMP), are three significant flavor compounds known particularly for their herbaceous and vegetative aromas. The methoxypyrazines (MPs) were first characterized in green bell peppers in 1969 [1] and were soon found to be widespread in plants and the key odorant in several raw crops such as asparagus, lettuce, potatoes, green beans, peas, peanuts, coffee and wine grapes (*Vitis vinifera*) [1-12]. MPs are of particular note because of their extremely low sensory detection thresholds, down to below 10 pg/g in water or wine [4, 5, 7] (Table 1). MP concentrations in some plants, particularly in vegetative tissue or unripe fruits, can exceed 1000 pg/g [10].

Table 1. Organoleptic properties of some 3-alkyl-2-methyoxypyrazines (ND = Not Determined).

	Detection Threshold in Water (ng/L)	Detection Threshold in Wine (ng/L)	Aroma Attributes
IBMP	2[a]	2 – 10[b,c,d]	Bell pepper[e], herbaceous[d], vegetal[f]
IPMP	1-2[e,g,h]	0.32 -2[c,i]	Bell pepper[e], raw potato[e], green peas[h], Earthy[g]
*s*BMP	1 [h]	ND	Green peas[h], galbanum oil[h]

a: [13]
b: [14]
c: [4]
d: [5]
e: [7]
f: [15]
g: [11]
h: [9]
i: [16]

1.2 Occurrence of Pyrazines in Nature

Pyrazines, alkylpyrazines and methoxypyrazines (Figure 1) are naturally occurring compounds found in plant and animal matter [10, 17, 18]. A biosynthetic pathway of pyrazines

in plants has since been suggested by Murray and Whitman, which involves the enzymatic condensation of an α-amino acid amide and 1,2-dicarbonyl [9]. Bacteria have also been shown to form pyrazines and alkylpyrazines from dicarbonyls and amino acids, and this process has been optimized for the commercial production of flavors for products such as nato or soy sauce [19-21]. Pyrazine and pyrazine derivatives are also known to be formed by Maillard reactions between an amino acid and sugars, particularly at high heat, during production of certain foods such as coffee beans that go through heat processing [17, 19, 22].

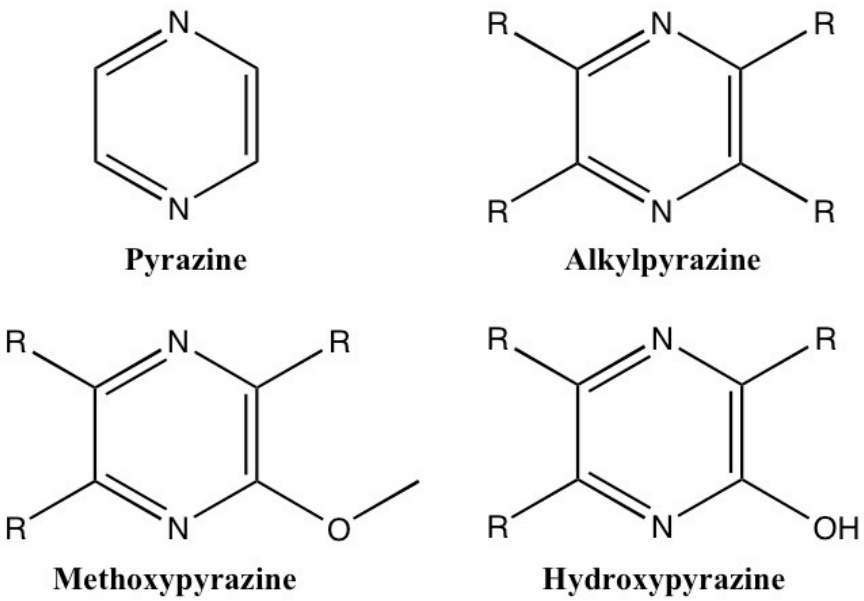


Figure 1. The structure of pyrazine and pyrazine derivatives.

There is very little known about biodegradation of pyrazines, beyond the hydroxylation of the pyrazine ring [23]. The ability of bacterial strains such as *Rhodoccocus erythropolis* to use 2,5-dimethylpyrazine as the sole source of both carbon and nitrogen suggests that there is some yet unidentified enzymatic reaction to degrade the pyrazine ring [18, 24].

Humans and other animals can oxidize pyrazines to either the corresponding alcohol or carboxylic acid by P450 type enzymes, or hydroxylating pyrazines by molybdenum-containing oxidases of the xanthin oxidase type [18, 23]. Pyrazines have also been found to have many bioactive effects, such as the pharmacological effects [18] or cellular effects such as changes in membrane fluidity [25], or the induction or inhibition of enzymes [26]. Even just the odor of pyrazines have been found to influence biological activity: leghorn chickens that were exposed to IBMP odors for 6 weeks were reported to lay larger eggs than the control group [27]. However, pyrazines on the whole have been found to be relatively non-toxic, with very high LD_{50} values in mice (at or over 2000 mg/kg) and have thus achieved GRAS (generally regarded as safe) status for use as flavoring agents in food [28].

1.3 Flavor Properties of Methoxypyrazines in Grapes and Wine

Alkyl-methoxypyrazines specifically have been found to play a great role in flavor chemistry both in raw foods as well as foods dependent on microorganism activity [1, 4, 8-10, 21, 29]. At a moderate level, IBMP plays an important role in the characteristic aroma in several varietals [4, 30]. However, the concentration of IBMP above detection threshold in wines quickly leads to unpleasant green and unripe aromas that are considered faulty [4, 30, 31]. The presence of IBMP is considered so key to the aroma composition of the varietal Sauvignon blanc, it has even been the subject of illegal addition to increase the concentration within finished wine from South Africa [32]. It has been determined that above 8-10 ng/L, IBMP has a significant correlation with a perceived vegetal aroma in both white and red Bordeaux Varietal wines, with an r^2 value of 0.551-0.74 [4, 5]. While this demonstrates a correlation between IBMP and herbaceousness, other green volatiles or masking effects are also likely important [4, 33-35].

1.4 Detection of Methoxypyrazines in Wine and Grapes

The first successful measurements of MPs in grapes via gas chromatography was reported by Bayonove et al in 1975 in Sauvignon blanc grapes [2]. The presence and aroma of IBMP in Sauvignon blanc was confirmed through the use of sensory analysis by gas chromatography effluent sniffing [2, 3, 36, 37]. This allowed for a new direction of studying MPs within wine grapes and wine.

Harris et al. in 1987 measured the IBMP concentration in Sauvignon blanc wine by GC/MS, which represented the first isolation and identification of MPs within wine [3]. Since then, the contribution of MPs to many of the other common herbaceous *Vitis vinifera* varietal aromas (such as those found in or with Cabernet sauvignon, Merlot, Cabernet franc and Carmenere) has been confirmed [4, 38-41]. Other MPs, such as 2-ethyl-3-methoxypyrazine and 3-methyl-2-methoxypyrazine, were originally thought to be important to wine aromas but were later dismissed as unimportant due to their high detection thresholds and low concentration within wine and wine grapes [3, 7, 37, 41]. IPMP and *s*BMP from grapes and wine were not typically found in levels high enough to be above detection threshold in most wines; thus IBMP was found to be the principle methoxypyrazine contributing to wine varietal aroma [4, 5, 30, 38, 41, 42].

1.5 Quantification of Methoxypyrazines

One of the major challenges to IBMP identification and quantification was due to the extremely low levels in grapes or wine. The introduction of using a labeled standard allowed the first accurate measurement of IBMP in wine [3]. The synthesis of deuterated standards offers several advantages for chemical and biochemical analysis, such as tracing transformations and behavior and the identification and quantification of compounds [43, 44]. Most trace volatile

analyses, including MP analyses, demand considerable sample pre-concentration and extraction, which risks inaccurate results due to samples losses. These risks can be limited in MP analyses by use of an isotopically labeled standard [3, 45-48]. It is well known that in gas chromatography changes in isotopic compositions and the position of the deuterium also result in changes in in retention time, due to slight changes in either the vapor pressure of the solute or the solute-stationary phase interaction [49-51]. In most cases, the replacement of a carbon-bound hydrogen with a deuterium changes the polarity [49, 52] or decreases the interaction with stationary phase, and thus decreases the retention time in comparison to the undeuterated compound [53]. This effect has been termed the "inverse isotope effect", or "chromatographic isotope effect" in gas chromatography-mass spectrometry (GC-MS) and is the most commonly reported behavior in gas or liquid [43, 50, 53]. The "normal isotope effect" then, is when the retention time of the deuterated compound increases in comparison to the undeuterated compound, i.e. when the lighter compounds elute first, is rarely seen outside of gas-solid chromatography at low temperatures [43, 50, 53]. A few studies have been done that show that changing the location of the deuterium on the compound can change the isotopic effect from normal to inverse, due to changes in vapor pressure or hydrophobicity among the isomers [50, 53-57]. In general, deuteration of methyl or methylene groups yields the inverse isotope effect [50, 54, 55].

1.6 Attempts to Control Methoxypyrazine Levels

With the determination of a correlation between IBMP and vegetal aromas in wine, focus shifted to the measurement and manipulation of IBMP within either the vineyard or the winery. As high IBMP concentrations can greatly affect the quality of a wine, the ability to remove or change IBMP concentrations was and is still of great interest.

There is a strong correlation between the level of IBMP concentrations in grapes at harvest and in the finished wine [31, 47], due to the relative inability of general cellar practices such as bentonite fining, pectinases and oak contact to affect IBMP concentration [58, 59] and the ease of extraction during the vinification process [36, 60]. In Sauvignon blanc grapes, IBMP was found in free run juice immediately after crushing, and was shown to have higher extraction in successive presses, with most of the IBMP extracted prior to alcoholic fermentation [36, 44]. IBMP also has the added challenge to winemakers of being chemically stable, as after three years of bottle aging there was no significant change in concentration [36]. Mitigating factors within the winery include thermovinification and settling of the wine, however these options are often not appropriate for particular styles of wine [36]. Activated charcoal has also been found to remove IBMP, but lacks selectivity and can remove desired compounds from the wine or juice [58].

Because of the challenge of selective removal of IBMP from juice or wine, research has also focused on factors that affect IBMP concentration in the vineyard. In wine grapes, it has been shown that IBMP levels increase preveraison, peaking two to three weeks before veraison then significantly decrease between veraison and harvest [36, 41, 60-63]. The maturity of grapes thus plays a strong role in the harvest concentration of IBMP, and so other factors such as growing degree days (GDD) will thus affect the final concentration [36, 41]. Several viticultural parameters have been examined for their effects on the concentration of IBMP. Vigor, crop load, and water status have all been shown to affect the concentration of IBMP in grapes at harvest, as the increase of peak IBMP has been correlated with rapid vine growth during pre-veraison, thus any viticultural practices that lead to increased vigor (such as lower crop loads, excess fertilization or high water status) lead to an increase in IBMP concentration [36, 64-66]. High

cluster light exposure has also been shown to lower the peak IBMP concentration though the concentrations at harvest are similar [61]. This suggests that IBMP is not degraded by light exposure, as it would be expected that exposed clusters at harvest would have much lower concentrations than those that were not exposed [61, 66].

1.7 Metabolism of Methoxypyrazines

As the variability between viticultural practices and the final concentration of IBMP grapes at harvest has yet to be fully explained, the most recent focus has been on the precursors and degradation products within the grape berry for an increased understanding in how viticultural practices affect IBMP. Although the biosynthetic pathway of IBMP has not yet been fully determined, it is hypothesized to be the product of the condensation of the amino acid leucine, that has gone through amidation, and glyoxal into 3-isobutyl-2(1H)-pyrazin-2-one, which along with its tautomer 3-isobutyl-2-hydroxypyrazine (IBHP), can then be methylated [7, 10, 67] (Figure 2). This pathway has been shown to occur in laboratory synthesis as well [7, 22, 45]. IBHP and other 3-alkyl-2-hydroxypyrazines (3-isopropyl-2-hydroxypyrazine [IPHP], 3-*sec*-butyl-2-methyoxypyrazine [*s*BHP]) are of interest as they have essentially no odor; most likely due to their low volatility and high polarity (Figure 1) [7].

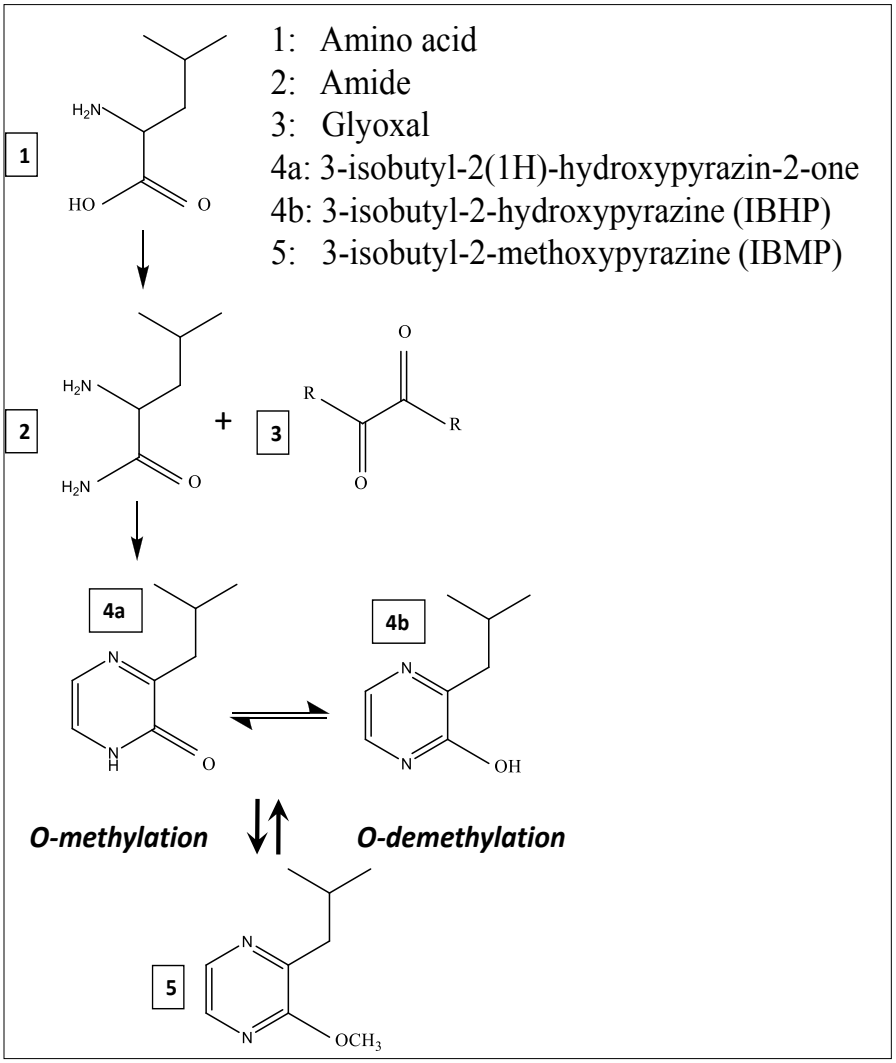


Figure 2. From Ryona et al. 2011, the putative biosynthesis and degradation pathways of IBMP through *O*-methylation and *O*-demethylation reactions [67].

The final IBMP biosynthesis step within grapes is thought to be an *O*-methylation of IBHP via an *O*-methyltransferase [10, 22]. Hashizume et al were the first to measure IBHP concentrations within wine grapes, and determined that it is found at levels similar to IBMP (at the pg/g level) [68]. They identified and purified an *S*-adenosyl-L-methionine (SAM) dependent *O*-methyltransferase (OMT) that was capable of methylating IBHP into IBMP [68, 69]. This OMT was the most effective against other substrates such as caffeic acid, but the purified protein had activity against a broad range of substrates including IBHP [69]. Upon further study, the OMT activity and the level of IBHP were positively correlated with the level of IBMP within different grape varieties at 40 days after anthesis; of particular note was the trace amount OMT activity in Pinot noir and low IBMP concentration, despite having measureable amounts of IBHP [68]. This offered another confirmation that the OMT is responsible for the last step of IBMP synthesis, as the trace activity of OMT resulted in low IBMP even in the presence of IBHP [68].

Dunlevy et al. in 2010 then identified the genes responsible for the production of two similar OMTs, *Vitis vinifera o*-methyltransferase 1 and 2 (VvOMT1, VvOMT2), and not only showed that recombinant *VvOMT1* and *VvOMT2* genes can methylate hydroxypyrazines but that the gene expression occurred during the same period as IBMP accumulation within the grapes [70]. They also found that these OMTs were capable of methylating a wide variety of substrates, which is in agreement with the previous findings by Hashizume [68, 70]. Interestingly, it was found that VvOMT1 and VvOMT2 had different activities regarding the methylation of IBHP and the similarly structured IPHP, and it was found that small changes in the active sites of VvOMT1 and VvOMT2 accounted for the difference and thus VvOMT1 is the more important OMT in IBMP synthesis [70, 71] (Figure 3).

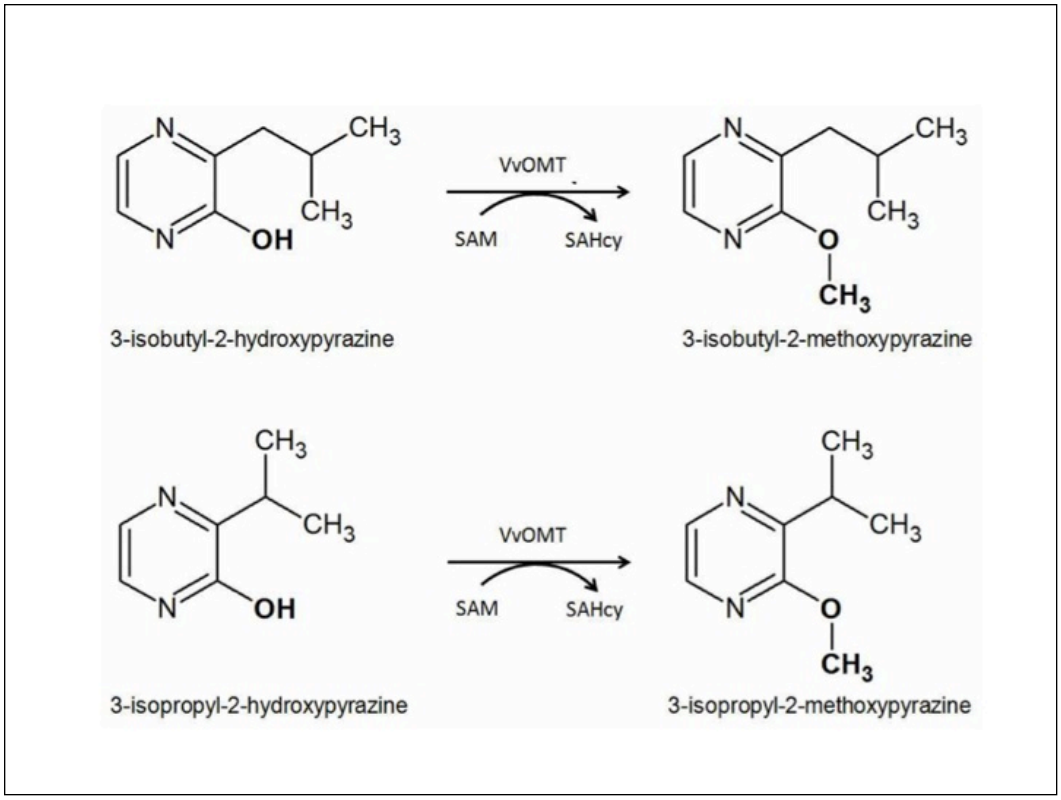


Figure 3. Proposed final step in the biosynthesis of IBMP and IPMP via VvOMTs in the presence of SAM, with a 3-alkyl-2-methoxypyrazine and *S*-adenosylhomocysteine as products, from Vallarino et al. 2011 and based on work from Hashizume et al 2001 [68, 71].

The degradation pathway of IBMP is as of yet unconfirmed, though it is hypothesized that it is enzymatically demethylated back into IBHP [67]. A study by Hawksworth et al. lends credence to this theory, as rats that were fed IBMP degraded it into IBHP, suggesting an *O*-demethylation degradation pathway [23]. In the most recent study by Ryona et al, it was determined that IBHP and IBMP were inversely correlated over ripening for both bell peppers and wine grapes, suggesting that IBHP is the first step in the IBMP degradation pathway [67] (Figure 2). It was also observed that acid treatment of Cabernet franc increased measured IBHP suggesting that some portion of IBHP existed as a glycoside ("bound" IBHP) [67].

1.8 Detection and Quantification of Hydroxypyrazines

Because IBHP appears to be both the precursor and degradation product of IBMP in grapes, determining factors that affect IBHP in the vineyard may be useful to understanding empirical observations regarding IBMP. However, IBHP has proven challenging to measure due to its relatively high polarity, low volatility, and low concentration, and the lack of suitable existing strategies for measuring IBHP has prevented much of its study within wine and wine grapes, [67, 68]. Previous approaches for measuring IBHP required either large amounts of juice (up to 1 L) or liquid extractions with large quantities of solvents that then required long evaporations to be able to concentrate IBHP to levels detectable by GC-MS [67, 68]. The method described by Hashizume et al (2001) was only able to detect a lower limit of 100 pg/g; and while the method by Ryona et al had a limit of detection of 25 pg/g it required the use of GCxGC-TOF-MS due to interferences [67, 68].

Silylation is a common method for derivatizing molecules that contain a proton capable of hydrogen bonding (e.g. –OH, =NH, $–NH_2$, –SH, –COOH), as the addition of the silyl groups result in decreased polarity and increased volatility allowing for better sensitivity and resolution for measurement via GC-MS [72]. Trimethyl silyl (TMS) derivatives have been found to be useful in the derivatization of hydroxyl groups (Figure 4), even those that are sterically hindered [23, 73, 74]. Sweeley et al determined that the combination of hydroxysilizane and trimethylchlorosilane derivatization compounds, along with pyridine, allowed for the complete conversion of sugars to *o*-tri-methyl silyl derivatives, allowing for measurement via gas chromatography [74].This was also shown to work for measurement of hydroxypyrazines by Hawksworth et al [23].

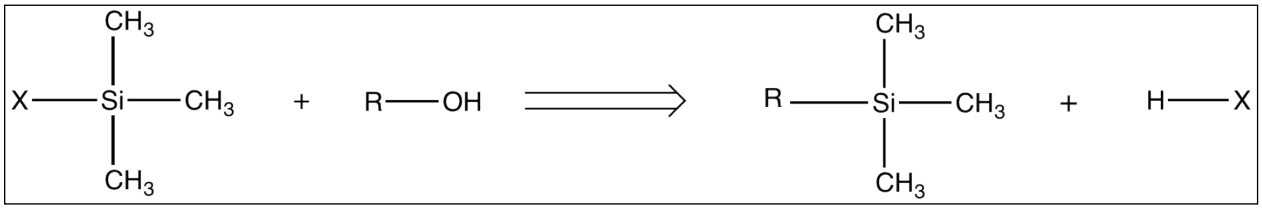


Figure 4. TMS derivatization reaction, where for TMCS: X = Cl and HMDS: X = NH-Si-$(CH_3)_3$ [72].

1.9 Goals of Project

Due to the importance of IBMP to wine flavor and acceptance, more research is necessary to understand its synthesis and degradation patterns. Very little previous work has been done in regards to understanding the concentration of the identified precursor, IBHP, in wine grapes and the correlation of IBHP to IBMP over the growing season. This is in part due to the challenge of measuring and quantifying the compound at such low concentrations. In this study, a method for isolation of IBHP via solvent phase extraction (SPE) and quantification via preparation of a stable deuterated standard, derivatization, and one dimensional gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) is presented, as well as data of IBHP and IBMP concentrations from throughout the 2010 and 2011 growing seasons. The fate of IBHP and IBMP during fermentation by different strains of wine yeast is also explored. This will help elucidate the role and relationship of IBHP in IBMP synthesis and degradation, both in the vineyard and in the winery, and could potentially lead to future understanding of how to control IBMP levels.

WORKS CITED

1. Buttery, R.G., Seifer, R.M., Guadagni, D.G., Ling, L.C., *Characterization of Some Volatile Constituents of Bell Peppers.* J. Agr. Food Chem., 1969. **17**(6): p. 1322-1327.

2. Bayonove, C.R., R. Cordonnier, and P. Dubois, *Etude d'une fractions caractéristique de arôme du raison de la variété Cabernet-Sauvignon; mise en evidence de la 2-méthoxy-3-isobutylpyrazine.* C. R. Acad. Sci. Paris. Ser. D, 1975. **281**: p. 75-78.

3. Harris, R.L.N., et al., *Determination of 2-Methoxy-3-alkylpyrazines in Wine by Gas Chromatography/Mass Spectrometry.* Vitis, 1987. **26**: p. 201-207.

4. Allen, M.S., et al., *Contribution of Methoxypyrazines to Sauvignon blanc Wine Aroma.* Am. J. Enol. Vitic., 1991. **42**(2): p. 109-112.

5. Roujou de Boubée, D., C. Van Leeuwen, and D. Dubourdieu, *Organoleptic Impact of 2-Methyoxy-3-isobutylpyrazine on Red Bordeaux and Loire Wines. Effect of Environmental Conditions on Concentrations in Grapes during Ripening.* J. Agr. Food Chem., 2000. **48**: p. 4830-4834.

6. Sala, C., J. Guasch, and F. Zamora, *Factors Affecting the Presence of 3-Alkyl-2-methoxypyrazines in Grapes and Wines. A Review.*, 2004, Universitat Rovira i Virgili. p. pp 53-58.

7. Seifert, R.M., Buttery, R.G., Guadagni, D.G., Black, D.R., Harris, J.G., *Synthesis of Some 2-Methoxy-3Alkylpyrazines with Strong Bell Pepper-Like Odors.* J. Agr. Food Chem., 1970. **18**(2): p. 246-249.

8. Friedel, P., et al., *Some Constituents of the Aroma Complex of Coffee.* J. Agr. Food Chem., 1971. **19**(3): p. 530-532.

9. Murray, K.E., J. Shipton, and F.B. Whitfield, *2-methoxypyrazines and the flavour of green peas (Pisum sativum.* Chemy Ind., 1970. **27**: p. 897-898.

10. Murray, K.E. and F.B. Whitfield, *The Occurrence of 3-Alkyl-2-methoxypyrazines in Raw Vegetables.* J. Sci. Fd. Agric., 1975. **26**: p. 973-986.

11. Parliment, T.H. and M.F. Epstein, *Organoleptic Properties of Some Alkyl-Substituted Alkoxy- and Alkylthiopyrazines.* J. Agr. Food Chem., 1973. **21**(4): p. 714-716.

12. Chetschik, I., M. Granvogl, and P. Schieberle, *Quantitation of Key Peanut Aroma Compounds in Raw Peanuts and pan-Roasted Peanut Meal. Aroma Reconstitution and Comparison with Commercial Peanut Products.* J. Agr. Food Chem., 2010. **58**: p. 11018-11026.

13. Buttery, R.G., et al., *Characterization of an important aroma component of bell peppers.* Chem. Ind., 1969. **12**: p. 490-491.

14. Kotseridis, Y., et al., *An analytical method for studying the volatile compounds of Merlot Noir clone wines.* Am. J. Enol. Vitic., 1998. **49**(1): p. 44-48.

15. Sala, C., et al., *Headspace solid-phase microextraction analysis of 3-alkyl-2methoxypyrazines in wines.* J. Chromatogr., 2002. **953**: p. 1-6.

16. Pickering, G.J., et al., *Determination of Ortho- and Retronasal Detection Thresholds for 2-Isopropyl-3-Methoxypyrazine in Wine.* J. of Food Sci., 2007. **72**(7): p. 468-472.

17. Hwang, H.-I., T.G. Hartman, and C.-T. Ho, *Relative Reactivities of Amino Acids in Pyrazine Formation.* J. Agric. Food Chem, 1995(43): p. 179-184.

18. Müller, R. and S. Rappert, *Pyrazines: Occurrence, Formation and Biodegradation.* Appl. Microbiol. Biotechnol., 2010. **85**: p. 1315-1320.

19. Besson, I., et al., *Pyrazine Production by Bacillus subtilis in solid-state fermentation on soybeans.* Appl. Microbiol. Biotechnol., 1997. **47**: p. 489-495.

20. Kim, K.-S., et al., *Optimum Conditions for the Production of Tetramethylpyrazine Flavor Compound by Aerobic Fed-batch Culutre of Lactococcus lactis subsp. lactis biovar. diacetylactis FC1.* J. Microbiol. and Biotech., 1994. **4**(4): p. 327-332.

21. Gallois, A., A. Kergomard, and J. Adda, *Study of the Biosynthesis of 3-Isopropyl-2-Methoxypyrazine Produced by Pseudomonas taetrolens.* Food Chem., 1988. **28**: p. 299-309.

22. Rizzi, G.P., *Formation of Methoxypyrazines in Reactions of 2(1H)-Pyrazinones with naturally Occuring Methylating Agents.* J. Agric. Food Chem, 1990. **38**: p. 1941-1944.

23. Hawksworth, G. and R.R. Scheline, *Metabolism in the Rat of Some Pyrazine Derivatives having Flavour Importance in Foods.* Xenobiotica, 1975. **5**(7): p. 389-399.

24. Rappert, S., et al., *Degradation of 2, 5-Dimethylpyrazine by Rhodococcus erythropolis Strain DP-45 Isolated from a Waste Gas Treatment Plant of a Fishmeal Processing Company.* Biodegradation, 2007. **18**: p. 585-596.

25. Nie, S.-Q., C.-Y. Kwan, and R.M. Epand, *Pyrazine Derivatives Affect Membrane Fluidity of Vascular Smooth Muscle Microsomes in Relation to their Biologic Acitivity.* European Journal of Pharmacology, 1993. **244**: p. 15-19.

26. Kim, S.G., et al., *Induction of Rat Liver microsomal Epoxide Hydrolase by Thiazole and Pyrazine: Hydrolysis of 2-Cyanoethylene Oxide.* Carcinogenisi, 1993. **14**(8): p. 1665-1670.

27. Barnea, A. and M. Rothschild, *The Odour of Pyrazine Increases the Egg Mass of Domestic Chickens (Gallys domesticus L.).* J. Zool. Lond., 2002. **257**: p. 411-416.

28. Adams, T.B., et al., *The FEMA GRAS Assessment of Pyrazine Derivatives Used as Flavor Ingredients.* Food and Chem. Tox., 2002. **40**: p. 429-451.

29. Gallois, A., A. Kergomard, and J. Adda, *Study of the Biosynthesis of 3-Isopropyl-2-Methoxypyrazine Produced by Pseudomonas taetrolens.* Food Chem, 1988. **28**: p. 299-309.

30. Allen, M.S., M.J. Lacey, and S. Boyd, *Determination of Methoxypyrazines in Red Wines by Stable Isotope Dilution Gas Chromatography-Mass Spectrometry.* J. Agric. Food Chem, 1994. **42**(8): p. 1734-1738.

31. Bogart, K. and L. Bisson, *Persistence of vegetal characters in winegrapes and wine.* Practical Vineyard and Winery, 2006. **86**: p. 13-20.

32. Rajchl, A., et al., *Methoxypyrazines in Sauvignon Blanc Wines, Detection of Addition of Artificial Aroma.* Czech J. Food Sci., 2009. **27**(4): p. 259-266.

33. Preston, L.D., et al., *Defining vegetal aromas in Cabernet Sauvignon using sensory and chemical evaluations.* Am. J. Enol. Vitic., 2008. **59**: p. 137-145.

34. Ebeler, S.E. and J.H. Thorngate, *Wine Chemistry and Flavor: Looking into the Crystal Glass.* J. Agr. Food Chem., 2009. **57**: p. 8098-8108.

35. Marais, J., *Sauvignon blanc Cultivar Aroma - A Review.* S. Afr. J. Enol. Vitic., 1994. **15**(2): p. 41-45.

36. Roujou de Boubée, D., *Research on 2-Methoxy-3-Isobutylpyrazine in Grapes and Wines.* 2003: p. Academie Amorim, Bordeaux.

37. Augustyn, O.P.H. and C.J. Van Wyck, *Some Volatile Aroma Components of Vitis vinifera L. cv. Sauvignon blanc.* S. Afr. J. Enol. Vitic., 1982. **3**(2): p. 53-60.

38. Hashizume, K. and N. Umeda, *Methoxypyrazine Content of Japanese Red Wines.* Biosci. Biotech. Biochem, 1996. **60**(5): p. 802-805.

39. Heymann, H., A.C. Noble, and R.B. Boulton, *Analysis of methoxypyrazines in wines, development of a quantitative procedure.* J. Agric. Food Chem, 1986. **34**: p. 268-271.

40. Calo, A., et al., *Caracterizazione di Cabernet franc e carmenère (Vitis sp.) e chiarimenti sulla loro coltura in Italia.* Riv. Vitic. Enol., 1991. **3**: p. 3-25.

41. Lacey, M.J., et al., *Methoxypyrazines in Sauvignon blanc Grapes and Wines.* Am. J. Enol. Vitic., 1991. **42**(2): p. 103-108.

42. Allen, M.S., et al., *Sauvignon blanc varietal aroma* Aust. Grapegrower & Winemaker, 1989. **292**: p. 51-56.

43. Matucha, M., et al., *isotope effect in gas-liquid chromatography of labelled compounds.* J. Chromatogr., 1991. **588**: p. 251-258.

44. Kotseridis, Y., et al., *Quantitative Determination of 2-Methoxy-3-Isobutylpyrazine in Red Wines and Grapes of Bordeaux Using a Stable Isotope Dilution Assay.* J. of Chrom. A, 1999. **841**: p. 229-237.

45. Gerritsma, D.A., et al., *Preperation of Labelled 2-Methoxy-3-alkylpyrazines: Synthesis and Characterization of Deuterated 2-Methyoxy-3-isopropylyrazine and 2-Methoxy-3-isobutylpyrazine.* J. Labelled Compd. Radiopharm., 2003. **4+**: p. 243-253.

46. Koch, A., et al., *2-Methoxy-3-isbutylpyrazine in Grape Berries and its Dependence on Genotype.* Phytochem., 2010. **71**: p. 2190-2198.

47. Ryona, I., B.S. Pan, and G.L. Sacks, *Rapid Measurement of 3-Alkyl-2-methoxypyrazine Content of Winegrapes to Predict Levels in Resultant Wines.* J. Agr. Food Chem., 2009. **57**: p. 8250-8257.

48. Sala, C., O. Busto, and J. Guasch, *Determination of 3-Alkyl-2-methoxypyrazines in Grapes, Musts and Wines: A Review.* 2008: p. Available at: http://www.tesisenxarxa.net/TESIS_URV/AVAILABLE/TDX-0626106-110827//18-PaperC3.pdf.

49. Bermejo, J., C.G. Blanco, and M.D. Guillén, *Gas chromatography of deuterated and protiated chloro derivatives of 1,4-dimethylbenzene.* J. of Chromatogr., 1986. **351**: p. 425-431.

50. Mráz, J., et al., *Unusual deuterium isotope effect on the retention of formamides in gas-liquid chromatography.* J. of Chromatogr., 1993. **641**: p. 194-198.

51. Cartoni, G.P., A. Liberti, and A. Pela, *Gas Chromatographic Separation of Polar Isotopic Molecules.* Anal. Chem., 1967. **39**(13): p. 1618-1622.

52. Bawaja, R., *Application of reversed-phase high-performance liquid chromatography for the separation of deuterium and hydrogen analogs of aromatic hydrocarbons.* Anal. Chim. Acta, 1987. **192**: p. 345-348.

53. Wade, D., *Deuterium isotope effects on noncovalent interactions between molecules.* Chemico-Biological Interactions, 1999. **117**: p. 191-217.

54. Kaliszan, R., *Quantitative Structure-Chromatographic Retention Relationships*1987, New York: Wiley.

55. Chizhkov, V.P. and L.A. Sinitsina, *"Impulseless" circulation gas chromatography of isotopic compounds.* J. Chromatogr., 1975. **104**(2): p. 327-335.

56. Van Hook, W.A., *Vapor Pressures of the Methylacetylenes, H3CCCH H3CCCD D3CCCH D3CCCD.* J. Chem. Phys., 1967. **46**(5): p. 1907-1918.

57. Wolff, H. and R. Wurtz, *Hydrogen Bonding and Vapor Pressure Isotope Effect of Dimethylamine.* J. Phys. Chem., 1970. **74**(7): p. 1600-1606.

58. Pickering, G.J., et al., *The evaluation of remedial treatments for wine affected by Harmonia axyridis.* . Int. J. Food Sci. Technol., 2006. **41**(1): p. 77-86.

59. Howell, G.S., J. Treloar, and R. Beaudry, *Cellar methods to reduce methoxypyrazine levels in Cabernet Franc and Cabernet Sauvignon wine*, 2006, Michigan State University.

60. Roujou de Boubée, D., et al., *Location of 2-Methoxy-3-isobutylpyrazine in Cabernet Sauvignon Grape Bunches and Its Extractability during Vinification.* Am. J. Enol. Vitic., 2002. **53**(1): p. 1-5.

61. Ryona, I., et al., *Effects of Cluster Light Exposure on 3-Isobutyl-2-methoxypyrazine Accumlulation and Degradation Patterns in Red Wine Grapes (Vitis vinifera L. Cv. Cabernet Franc).* 56, 2008: p. 10838-10846.

62. Sala, C., et al., *Influence of Vine Training and Sunlight Exposure on the 3-Alkyl-2-methoxypyrazines Content in Musts and Wines from the Vitis vinifera Variety Cabernet Sauvignon.* J. Agr. Food Chem., 2004. **52**: p. 3492-3497.

63. Hashizume, K. and T. Samuta, *Grape Maturity and Light Exposure Affect Berry Methoxypyrazine Concentration.* Am. J. Enol. Vitic., 1999. **50**(2): p. 194-198.

64. Sala, C., et al., *Contents of 3-alkyl-2-methoxypyrazines in musts and wines from Vitis vinifera variety Cabernet Sauvignon: influence of irrigation and plantation density.* J. Sci. Fd. Agric., 2005. **85**: p. 1131-1136.

65. Scheiner, J., et al., *Modeling Impacts of Viticultural and Environmental Factors on 3-Isobutyl-2-methoxypyrazine in Cabernet franc Grapes.* Am. J. Enol. Vitic., 2012. **63**(1): p. 94-105.

66. Scheiner, J.J., G.L. Sacks, and J.E. Vanden Heuvel, *How Viticultural Factors Affect Methoxypyrazines*, in *Wines & Vines*2009.

67. Ryona, I., S. Leclerc, and G.L. Sacks, *Correlation of 3-Isobutyl-2-methoxypyrazine to 3-Isobutyl-2-hydroxypyrazine during Maturation of Bell Pepper (Capsicum annum) and Wine Grapes (Vitis vinifera).* J. Agr. Food Chem., 2010. **58**: p. 9723-9730.

68. Hashizume, K., et al., *S-Adenosyl-L-methionine-dependent O-Methylation of 2-Hydroxy-3-alkylpyrazine in Wine Grapes: A Putative Final step of Methoxypyraxzine Biosynthesis.* Biosci. Biotech. Biochem., 2001. **65**(4): p. 795-801.

69. Hashizume, K., et al., *Purification and Characterization of a O-Methyltransferase Cabaple of methylating 2-Hydrozy-3-alkylpyrazine from Vitis viniferai L. (cv. Cabernet Sauvignon).* Biosci. Biotech. Biochem., 2001. **65**(10): p. 2213-2219.

70. Dunlevy, J.D., et al., *Two O-methyltransferases Involved in the Biosynthesis of Methoxypyrazines: Grape-Derived Aroma Compounds Important to Wine Flavor.* Plant Mol. Biol., 2010. **74**: p. 77-89.

71. Vallarino, J.G., et al., *Biosynthesis of Methoxypyrazines: Elucidating the Structural/Functional Relationship of Two Vitis vinifera O-Methyltransferases Capable of Catalyzing the Putative Last Step of the Biosynthesis of 3-Alkyl-2-Methoxypyrazine.* J. Agric. Food Chem, 2011. **59**: p. 7310-7316.

72. Knapp, D.R., *Handbook of Analytical Derivatization Reactions*1979, New York: John Wiley & Sons.

73. Shareef, A., M.J. Angove, and J.D. Wells, *Optimization of Silylation Using N-methyl-N-(trimethylsilyl)-trifluoroacetamide, N,O-bis-(trimethylsilyl)-trifluoroacetamide and N-(tert-butyldimethlsilyl)-N-methyltrifluoroacetamide for the Determination of the Estrogens Estrone and 17α-ethinylestradiol by Gas Chromatography-Mass Spectrometry.* J. of Chrom. A, 2006. **1108q**: p. 121-128.

74. Sweeley, C.C., et al., *Gas-Liquid Chromatography of Trimethyl Derivatives of Sugars and Related Substances.* J. Am. Chem. Soc., 1963. **85**: p. 2497-2507.

CHAPTER 2

QUANTIFICATION OF 3-ISOBUTYL-2-HYDROXYPYRAZINE, A KEY INTERMEDIATE IN IBMP METABOLISM

Abstract

3-isobutyl-2-hydroxypyrazine (IBHP) is thought to be a key intermediate in both the biosynthesis and degradation of the herbaceous smelling 3-isobutyl-2-methoxypyrazine (IBMP), but quantitative methods for IBHP analysis are not widely reported and its behavior over the growing season is not well understood. An improved method for quantification of IBHP was developed, using a $^{2}[H_2]$-IBHP standard, isolation by mixed-mode cation exchange SPE and silylation prior to GC-TOF-MS analysis. A limit of detection of 33 ng/L could be achieved for a 100 ml juice sample. This method was used to quantify IBHP during the 2010 growing season at sites in both the New York Finger Lakes and the California Central Valley regions. In New York Cabernet franc, the concentration of free IBHP increased to over 280 pg/berry and in California Merlot IBHP peaked to over 470 pg/berry. Peak concentrations of free IBHP appeared to correlate with peak concentrations of IBMP. The IBHP degradation occurred at least 2 weeks after typical IBMP synthesis and degradation, though it appears as though different varietals and clones have differing degradation patterns. Acid-labile IBHP in Cabernet franc degraded following veraison levels nearing 950 pg/berry, to levels statistically indistinguishable from free IBHP near harvest. The ability of several yeast strains to methylate IBHP into IBMP was examined, and it was not found under normal fermentation conditions for the identified yeast to synthesize IBMP from IBHP.

Introduction

The 3-alkyl-2-methoxypyrazines (MPs), including 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-3-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (*s*BMP) contribute herbaceous and vegetative aromas to many plant-derived foods. The MPs were first characterized in green bell peppers [1] and were subsequently found in several other plants including asparagus, lettuce, potatoes, green beans, peas, and wine grapes (*Vitis vinifera*) [1-3]. MPs possess low sensory detection thresholds of <10 pg/L in water or wine [4-6], and their concentrations in vegetative tissue and unripe fruits can exceed 1000 pg/g [3].

The MPs, and particularly IBMP, are known to play an important role in the flavor of some wines [5-10], including varietals such as Sauvignon blanc, Cabernet Sauvignon, and Cabernet franc [5, 6, 10-12]. While modest concentrations may contribute positively to varietal character, IBMP concentrations well above threshold may result in unacceptable green and unripe aromas [5, 10, 11]. The effects of several environmental factors on IBMP accumulation and degradation have been investigated, including cluster shading, water availability, and nitrogen fertilization [13-17]. However, interpretation of these empirical results is often challenging. For example, several authors have observed that pre-veraison cluster shading in the vineyard results in increased accumulation of IBMP [13, 14, 16], but a biochemical explanation for this phenomenon is not available.

Interpretation of these results should be facilitated by understanding the behavior of metabolic intermediates of MPs. MP biosynthesis in plants has not yet been entirely determined. It is hypothesized to begin with the condensation of NH_3, an appropriate amino acid (e.g. leucine, valine) and glyoxal to form a 3-alkyl-2(1H)-pyrazin-2-one and its tautomer 3-alkyl-2-hydroxypyrazine (HP) [3, 4, 12]. Subsequently, HPs are thought to be *O*-methylated to form

MPs [3, 18]. An *S*-adenosyl-L-methionine (SAM) dependent *O*-methyltransferase (OMT) capable of methylating IBHP into IBMP has been identified and purified [19, 20]. More recently, two genes (*VvOMT1*, *VvOMT2*) have been cloned and shown to be capable of methylating HPs. Transcription analysis revealed that these genes are expressed pre-veraison, corresponding with the time of maximum IBMP accumulation within the grapes [21]. The degradation pathway of IBMP in plants is not as well studied. A recent report has suggested that IBMP may be demethylated to reform IBHP and then partially glycosylated [12], similar to metabolism of IBMP observed in rats [22].

Because IBHP appears to be both the precursor and degradation product of IBMP in grapes, characterizing the relationship of IBMP and IBHP during the growing season should assist in interpretation of empirical viticultural studies. For example, it is not known if the elevated IBMP accumulation observed in shaded fruit results from increased production of IBHP, decreased expression of *VvOMT1* and *VvOMT2*, or some other factors. However, only a couple of reports on IBHP in grapes exist [12, 20], and both consider only a limited number of time points. In part, this may reflect analytical difficulties, since IBHP's amphiphilic nature, low volatility, and low concentration make it a challenging analyte for GC-MS.

In this study, we describe an improved method for IBHP quantification based on mixed mode solvent phase extraction (SPE) in the presence of a deuterated standard, silylation, and gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) of the derivatized IBHP. We then present data on the correlation of IBHP and IBMP concentrations in grapes over two growing seasons across multiple sites. Lastly, the ability of IBHP and IBMP to interconvert during alcoholic fermentation was examined, as an study of the ability of other OMTs to methylate IBHP.

Materials and Methods

Chemical Reagents and Standards

3-Isobutyl-2-hydroxypyrazine (IBHP) was purchased from Manchester Organics Ltd. (97%, Sutton Weaver, U.K.). $^{2}[H_2]$-3-isobutyl-2-hydroxypyrazine was synthesized as described below. $^{2}[H_2]$- 3-isobutyl-2-methoxypyrazine was synthesized as described elsewhere [23]. Sodium chloride (NaCl), potassium carbonate, sodium hydroxide (NaOH), D-glucose, pyridine (99%), hexamethyldisilyl (HMDS), manganese sulfate, 3-isobutyl- 2-methopyrazine (99%), molecular sieve UOP size 3A, glycol bis(sodium bisulfite), deuterium oxide (D_2O, 99%) and L-leucinamide hydrochloride (99%) were purchased from Sigma Aldrich (Allentown, PA), ethylenediaminetetraacetic acid (EDTA), citric acid, ascorbic acid, sodium chloride (NaCl), ethanol, and trimethylsilyl chloride (TMCS) were purchased from Acros Organics N.V. (Geel, Belgium), ethyl acetate and acetonitrile were obtained from VWR International (West Chester, PA), and dichloromethane, ammonium hydroxide, calcium chloride, tartaric acid, hydrochloric acid (37%), methanol, dichloromethane, magnesium sulfate and D-fructose were purchased from Fisher Chemical (Fairlawn, NJ). YPD Agar and YPD Broth were purchased from B.D. Difco (Franklin Lakes, NJ). Deionized, distilled water was obtained from a Milli-Q purification system Millipore (Billerica, MA).

$^{2}[H_2]$-3-Isobutyl-2-Hydroxypyrazine Synthesis

$^{2}[H_2]$-3-Isobutyl-2-hydroxypyrazine synthesis was by adaptation of previous methods [24, 25]. Glyoxal bis(sodium bisulfite) (2.65 g) was refluxed with 10 ml of D_2O in a 50 ml round bottom flask at 100°C for 24 hours to yield a white crystalline solid in a yellow liquid, $^{2}[H_2]$-

glyoxal [24], which was used in the next step without further purification. In a separate round bottom-flask, leucinamide hydrochloride (0.166 g) was dissolved in 2 ml of methanol and cooled to -35°C. The $^{2}[H_2]$-glyoxal slurry (0.174g) was added and stirred vigorously. Aqueous NaOH (12M, 200 μL) was added dropwise over the course of 20 minutes. The solution was warmed to room temperature, and stirred continuously for 2 hours. The mixture was cooled to 0°C, acidified with 200μL of 12 M HCl followed by addition of 0.2 g of sodium carbonate. The mixture was filtered and 2 mL of water was added to the filtrate. The methanol was removed by evaporation under reduced pressure, and the $^{2}[H_2]$-3-isobutyl-2-hydroxypyrazine was extracted from the aqueous layer with 3x5ml aliquots of dichloromethane. Following silylation, the following GC-MS spectra was observed [*m/z* (RI%)]: 153 (19), 169 (88), 184 (100), 211 (28), consistent with a 2 a.m.u. shift as compared to the IBHP spectra presented in Hawksworth, et al [22].

Fruit Samples for Time Course Studies of IBMP and IBHP

Detailed studies on the concentration of IBHP and IBMP throughout the growing season were performed over 2010 in both the Finger Lakes region of New York State and the Central Valley region of California. Information on the sites and cultivars used in the time course study can be found in Table 2. For sampling, 1 kg of grape clusters were randomly selected from throughout a block and frozen at -4 °C prior to analysis. Samples were taken at either weekly or biweekly intervals from fruit set until harvest.

Table 2. Time course cultivar data.

Sample	Location	Cultivar/Clone	Veraison Date	Harvest Date
CF4	Cornell University Experimental Vineyards, Cayuga Lake AVA, New York	Cabernet franc Clone 4	August 15	October 12
CF1	Cornell University Experimental Vineyards, Cayuga Lake AVA, New York	Cabernet franc Clone 1	August 15	October 12
ML	Constellation Brands Vineyards, Central Valley AVA, California	Merlot	July 15	September 9

Grape Juice Preparation

Frozen grapes (400 g) were thawed, manually destemmed, and homogenized with a Waring Blender (model 5011, Torrington, CT) at low speed for 1 minute in the presence of 50 mg/kg ascorbic acid. The homogenate was pressed through cheesecloth and the juice collected. Because we had previously observed that IBHP is well extracted into the juice under these conditions [12], the insoluble solid material was discarded. An aliquot of juice was separated for measurements of pH, TA, and soluble solids. The filtered juice was loaded into either 250 or 500 ml NALGENE polycarbonate centrifuge bottles (VWR International, West Chester, PA) and centrifuged at 9000 rpm for 30 min at 4°C (Sorvall RC6+ Centrifuge, Thermo Scientific, Waltham, MA). The supernatant was filtered through Whatman No. 4 filter paper and the resulting clarified juice was stored at -10°C until needed. Preparation of juice samples and subsequent preparation steps were performed in duplicate for each sample.

Basic Juice Chemistry Measurements

Soluble solids were measured on extracted juice by digital refractometer (Leica Auto ABBE; AO Scientific Instruments, Buffalo, NY). The titratable acidity (TA) and pH were measured with an automatic titrator (Titrino Plus 848 Doser, 869 Autosampler, Methrohm USA Riverview, FL and Accumet Excel XL25 pH meter, Thermo Fisher Scientific, Waltham, MA). The TA was measured via 5 ml aliquot against 0.1 N NaOH to a pH of 8.2.

IBHP Extraction via Cation-Exchange SPE Method

Solid phase extraction (SPE) of IBHP from grape juice was performed on a Varian 24-cartridge positive pressure manifold (Palo Alto, CA), using 6 mL cartridges packed with 200 mg of Bond Elut Plexa PCX sorbent (Agilent, Santa Clara, CA). Juice samples (100 mL) were spiked with $^{2}[H_2]$-IBHP to yield a final concentration of 500 ng/L and adjusted to pH = 2 with HCl. Cartridges were conditioned with 5 mL dichloromethane, 10 mL methanol and 20 mL of water prior to sample loading. Each sample was split into two 50 mL sub-samples and extracted in parallel on two identically conditioned cartridges to expedite sample processing. Following sample loading, each cartridge was washed with 6 mL of 5% v/v methanol solution adjusted to pH = 2 with HCl and the cartridge dried with N_2 (25 psi) for 30 minutes. IBHP was eluted with 3 ml of 2% ammonium hydroxide in ethyl acetate/dichloromethane (4:1 v/v). Subsample extracts were recombined and evaporated to dryness under N_2.

Derivatization of SPE Extracts:

Silylation was adapted from the methods of Hawksworth et al [22] and Sweeley et al [26]. The dried extract was reconstituted in 0.5 mL of pyridine, dried over a molecular sieve, and 100 μL of HMDS and 50 μL of TMCS were added. A small amount of gas, likely H_2, was formed upon addition of the TMCS, and the reaction immediately became cloudy, likely due to the precipitation of a chloride salt [22]. The solution was heated for 20 min at 65°C, cooled, analyzed by GC-TOF-MS without further extraction of the reaction mixture.

Quantification of Derivatized IBHP Extracts by GC-TOF-MS

The derivatized IBHP was quantified by gas chromatography-time-of-flight mass spectrometry (Pegasus, LECO Corp., St. Joseph, MI). The GC system was a comprehensive 2-D GC (GCxGC), operated in one-dimensional mode by turning off the cryomodulator and setting the secondary oven temperature to +20 °C as compared to the primary oven. The GC column was a DB-FFAP (25 m x 0.25 mm x 0.25 μm, Agilent) coupled to a DB-17 (2 m x 0.1 mm x 0.2 μm, Agilent) via an inert glass press-tight connector. Three μL were injected, splitless, into an injector operated in pulsed splitless mode and held at 250°C. Helium was used as a carrier gas at a flow rate of 1 mL/min. The oven was initially at 70 °C and held for 5 min, then ramped at 6.3 °C/min to 240 °C with a 8 min final hold. The MS transfer line temperature was 260 °C. The TOF-MS was operated in EI mode, with an ionization energy of -70 eV. Data processing was carried out by the LECO ChromaTOF software. The qualifier ions for IBHP were *m/z* 151, 167, 209, and the quantifier ion was *m/z* 182. The qualifier ions for $^2[H_2]$-IBHP were *m/z* 153, 169, 211 and the quantifier ion was *m/z* 184. Quantification was performed with respect to appropriate calibration curves, described below.

Calibration Curve and Limits of Detection for IBHP

Calibration curves for IBHP were prepared using model juice (10% w/v fructose, 10% w/v glucose, 7.5 % w/v tartaric acid, pH=3.5) at concentrations of 0, 100, 250, 500 and 1000 ng/L. The limit of detection (LOD) was defined as the minimum peak area necessary to achieve a signal-to-noise ration of 3:1 and was estimated from calibration curves using Pallesen's method [27].

Recovery Experiments

To determine IBHP recovery by the SPE extraction protocol, 100 mL of model juice was spiked with 50 ng $^{2}[H_2]$-IBHP and extracted by the SPE protocol described above. Unspiked controls were also extracted by the same SPE protocol, and the SPE eluent spiked with 50 ng $^{2}[H_2]$-IBHP prior to derivatization. Triplicates of both spiked juice and spiked reference samples were analyzed. Recovery was calculated as the ratio of the signal achieved when IBHP was spiked before SPE extraction divided by the signal achieved when IBHP was spiked after extraction.

Analysis of Total IBHP by Acid Hydrolysis

Time course samples from Clone 4 were evaluated for acid-releasable IBHP. Juice samples were acidified to pH 2 by addition of HCl, and $^{2}[H_2]$-IBHP was added to yield a final concentration of 500 ng/L. The acidified juice was incubated in a water bath (100˚C, 1 h). IBHP was then extracted from the hydrolyzed sample using the same protocol as described for free IBHP.

Quantification of IBMP

IBMP in grapes and wine samples was quantified by GCxGC-TOF-MS using a method described elsewhere [28]. This previous method was also adapted for quantification of IBMP in resting cell experiments with the following modifications. For the experiments with 1 μg/L IBMP added, 1 ml of supernatant was diluted to 10 ml with MilliQ-H_2O and $^2[H_2]$-IBMP was added to a concentration of 100 ng/L. For the resting cell experiment with no added IBMP, 1 ml of supernatant was diluted to 2 mL with MilliQ-H_2O, and 20 ng/L of $^2[H_2]$-IBMP was added along with 0.6 g of NaCl. The SPME incubation time was increased to 45 minutes to compensate for the reduced sample volume. Calibration curves were made from the tartrate buffer with either 100 ng/L $^2[H_2]$-IBMP or 20 ng/L $^2[H_2]$-IBMP.

Conversion of IBHP to IBMP in Small Scale Fermentations

The fermentation protocol was adapted from Fia et al [29]. Commercial pasteurized, concentrated Pinot gris grape juice was purchased (Let's Do Wine, letsdowine.com) and reconstituted as per manufacturer's instructions to 20˚Brix, 6.5 g/L titratable acid pH=3.6. Four commercial *S. cerevisiae* strains, EC1118, Vin13, AMH and CY3079 (Lallemand Inc, Italy) and one non-*S.cerevisiae* strain, *Kloeckera apiculata* (isolated in the lab of R. Mira de Orduña), were inoculated into 150 ml of juice from YPD agar plates, and incubated at 25 ˚C for 48 hours and 72 hours for the *S. cerevisiae* strains and *K. apiculata* respectively, and in turn used to inoculate 600 ml of juice in a 1-L Corning Pyrex media storage bottles fitted with fermentation locks. IBHP was added to yield a concentration of 1 μg/L, and fermentations were carried out in triplicate. The flasks were incubated at 20˚C and shaken at 100 rpm on a gyratory shaker through fermentation. Fermentation kinetics were tracked by measured change in mass of the container.

Must samples (10 mL) were taken at 2-6 day intervals for analysis of IBMP. Samples were taken quickly via pipette to prevent excess oxygen introduction. Fermentation was considered complete either when fermented to dryness (<0.5% R.S.) or when there was no change in weight over 5 days.

Conversion of IBHP to IBMP by Resting Cells

Resting cell experiments were based on the method of Fia et al. [29]. EC1118 yeast cells grown on a YPD plate were inoculated into 100 mL of YPD media for 24 h at 25˚C, then 1 mL was inoculated into 600 mL YPD media for 48 h at 25˚C. Two hundred mL of this media were centrifuged at 3900 rpm for 3 min at 25 ˚C, and the supernatant was removed. Cells were washed with a tartrate buffer (7.5 g/L tartaric acid, 1.0 g/L $MgSO_4 \cdot 7H_2O$ and 0.25 g/L $MnSO_4 \cdot 4H_2O$, adjusted to pH 3.5 with NaOH), and resuspended in 20 mL of the buffer. The cell suspension was pipetted into a glass vial and placed in a water bath at 20 ˚C. Each experiment was performed in duplicate. IBHP (1.μg/L) or IBHP and IBMP (both at 1 μg/L) were added to the suspensions. Samples (1.5 mL) were taken after 15, 30, 45, 60, and 120 min, and immediately frozen at -80˚C. Before IBHP or IBMP analysis, samples were thawed and centrifuged at 3500 rpm.

Statistical Analysis

Statistical analysis was performed by JMP version 8 (SAS Institute, Cary, NC) using paired Student's *t* test and least squares model fit.

Results and discussion

Synthesis of $^{2}[H_2]$-IBHP Internal Standard

The $^{2}[H_2]$-IBHP internal standard was performed by the sequence shown in Figure 5, adapted from earlier protocols by Bertz and by Gerritsma et. al [24, 25]. Figure 6A and 6B show the mass spectra of the qualifying ions for underivatized, while figures 7A and 7B show the spectra of the qualifying ions for derivatized IBHP and $^{2}[H_2]$-IBHP . The deuterated standard was stable in acetonitrile solution at 0°C for >12 months. The mass spectrum of the $^{2}[H_2]$-IBHP standard is consistent with a 2 amu shift as compared to the unlabeled standard. The latter spectrum is consistent with that of Hawksworth, et al [22].

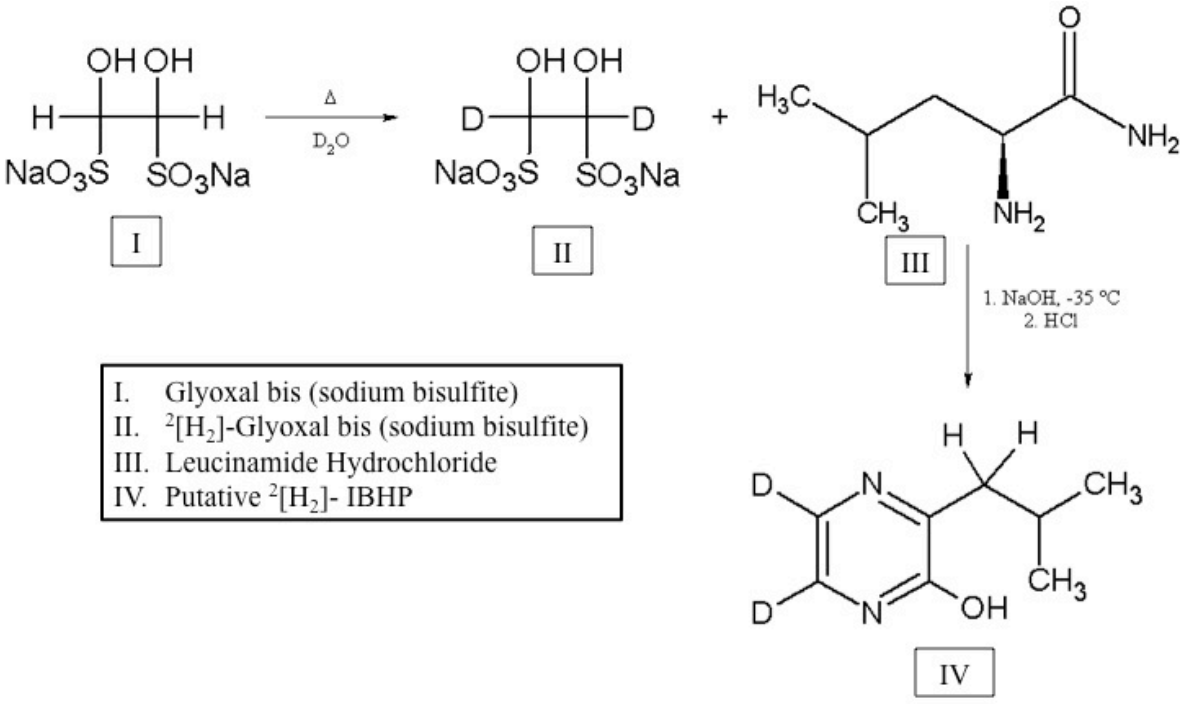


Figure 5. Synthesis and putative structure of $^{2}[H_2]$-IBHP.

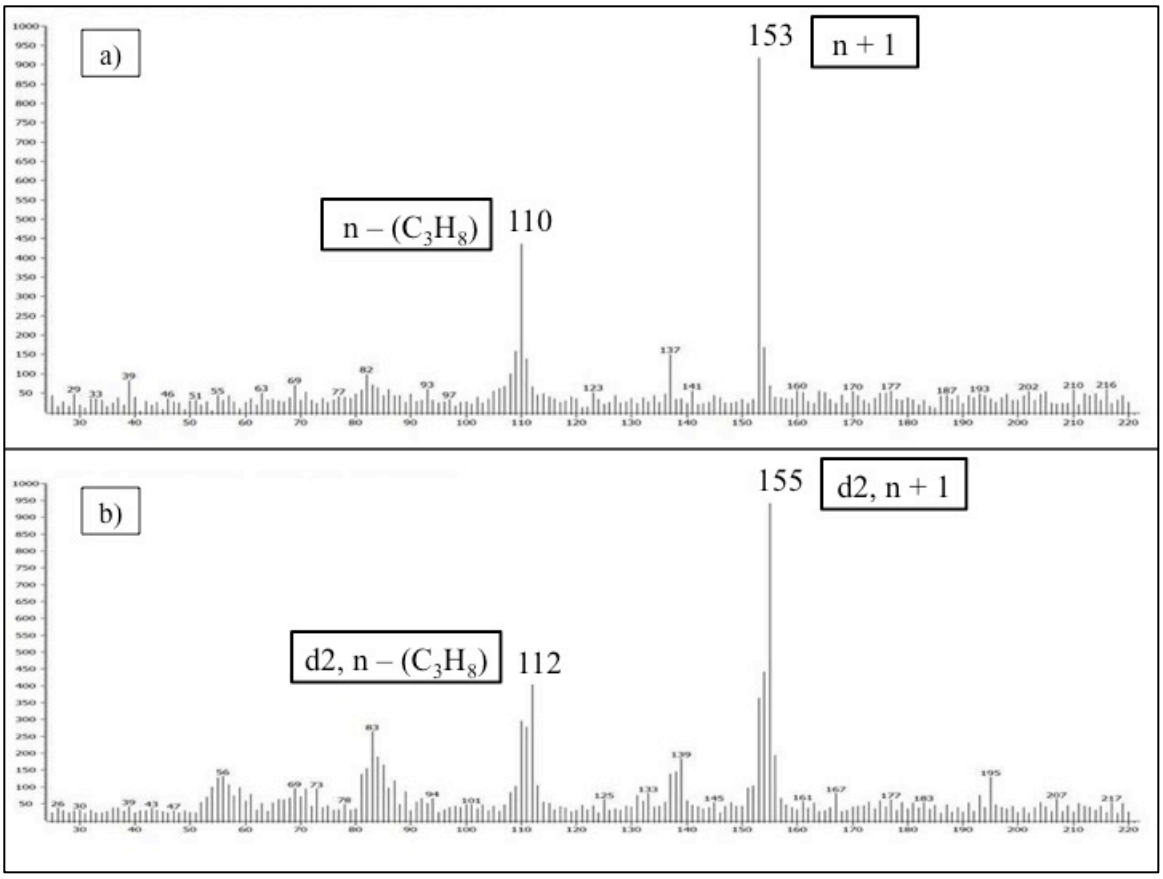


Figure 6. GC/CI-MS spectra displaying qualifying ions of a) 1 µg/L IBHP and b) 1 µg/L synthesized $^{2}[H_2]$-IBHP

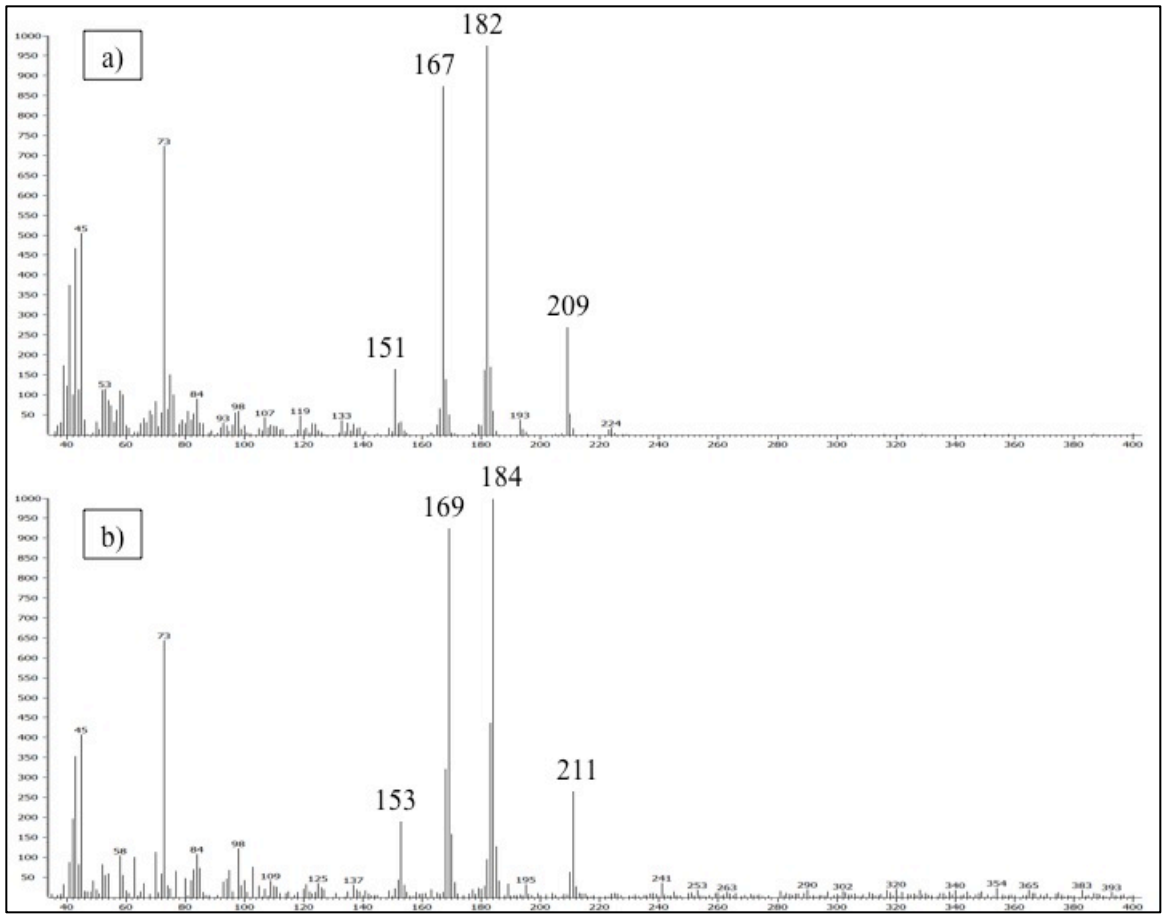


Figure 7. GC-TOF-MS mass spectra displaying qualifying ions of the derivatization products of a) 500 pg/L IBHP and b) 500 pg/L synthesized $^{2}[H_2]$-IBHP.

SPE and Derivatization Method – Optimization and Figures of Merit

An optimized protocol for isolating the basic IBHP analyte by mixed-mode cation exchange was developed using Bond Elut Plexa PCX SPE columns. A similar approach has been used for isolating MPs from wine and was used as a starting point [30]. In our work, the juice was initially acidified with HCl to pH = 2 prior to loading as opposed to the H_3PO_4 used in the previous report, as the latter resulted in a sizable silylated phosphoric acid interference following derivatization (data not shown). The wash step used only 5% methanol: water (v/v), pH 2, as higher methanol content resulted in sensitivity losses without corresponding improvement in

interference removal. Ethyl acetate was chosen as the eluent, with 20% DCM (v/v) made basic by the addition of 2% NH_4 as it optimized the amount of IBHP removed from the column while minimizing the interferences eluted. The SPE step resulted in a recovery of 92.8 of IBHP (SE = 3.18).

Earlier studies of IBHP in grapes performed GC-MS analyses on underivatized IBHP [12, 20], but this results in poor chromatographic behavior due to the presence of multiple H-bonding sites on IBHP and required much higher concentrations. We attempted to derivatize IBHP with multiple reagents, including trifluroacetic acid (TFAA), HMDS, and Sweeley's reagent. TFAA and HMDS alone resulted in incomplete derivatization of IBHP. Sweeley's reagent, composed of HMDS and TMCS in pyridine, has been previously recommended for derivatization of IBHP [22]. Direct injection of the reaction mixture showed a single derivative and no evidence of the IBHP starting material. As a precaution, silylation IBHP is unstable at room temperature, and no silylated peak could be detected after storing derivatized samples for 12-24 h at 25°C. However, it was possible to re-derivatize samples without affecting accuracy if this degradation occurred.

GC-TOF-MS chromatograms of the *m/z* 182 and 184 quantifying ions of silylated IBHP and its deuterated analogue run on a DB-FFAP column are shown in Figure 8. Figure 8A, 8B and 8C show chromatograms of model juice spiked with 0, 500, and 1000 ng/L IBHP, respectively, following extraction and derivatization. Figure 8D shows a veraison sample of Cabernet franc, containing approximately 200 ng/L of native IBHP. All samples contain 500 ng/L $^{2}[H_2]$-IBHP internal standard. While the majority of samples were run on an FFAP column, we observed an interference for samples collected at the latest time point. Therefore, samples from the last time points were run on an RTX-50 column (50% phenyl) to avoid these interferences. Interestingly, the silylated derivatives exhibited a normal isotope effect on the FFAP column (Figure 8), in

which the heavier deuterated compound elutes after the undeuterated analog. This was not the case for the 50% phenyl column, which exhibited the more common inverse isotope effect.

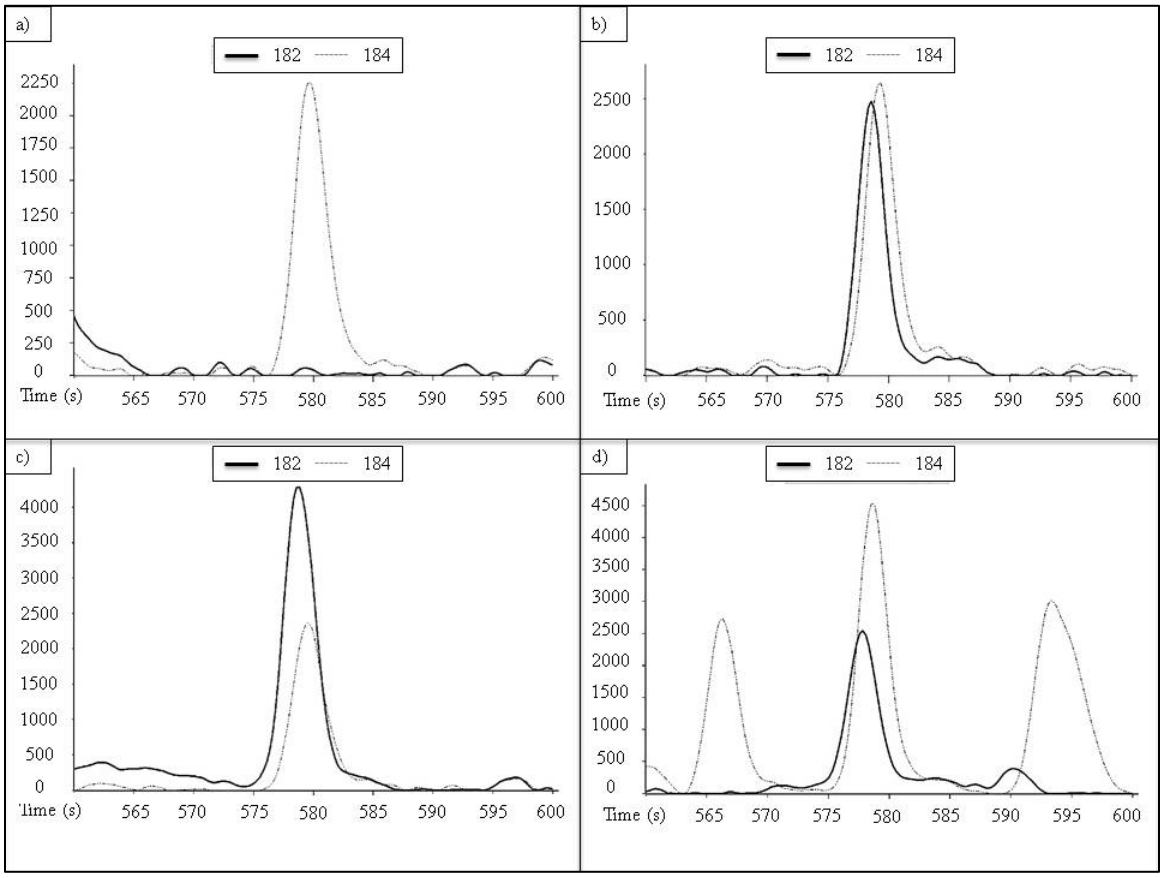


Figure 8. GC-TOF-MS chromatograms displaying m/z 182 and 184 ions of derivatized **a)** model juice spiked with 0 ng/L IBHP and 500 ng/L $^{2}[H_2]$-IBHP, **b)** 500 ng/L IBHP with 500 ng/L $^{2}[H_2]$-IBHP, **c)** 1000 ng/L IBHP and 500 ng/L $^{2}[H_2]$-IBHP and **d)** Cabernet franc juice at veraison spiked with 500 ng/L $^{2}[H_2]$-IBHP.

Method Linearity and Limits of Detection

Five point calibration curves were prepared in model juice at 0, 100, 250, 500, and 1000 ng/L oh IBHP. Good linearity as the internal standard resulted in a linear calibration curve (R^2 = 0.995, %RMSE = 5.7). The limit of detection for IBHP was estimated from Pallensen's method as 35 ng/L in model juice, and was defined as the minimum peak area necessary for a signal-to-

noise ratio of 3:1 [27]. The limits of detection in true juice samples were lower and estimated to be 20 ng/L, as at concentrations of 22 ng/L we saw %RSD of 9, and at 32 ng/L we saw %RSD of 16, which is within acceptable limits.

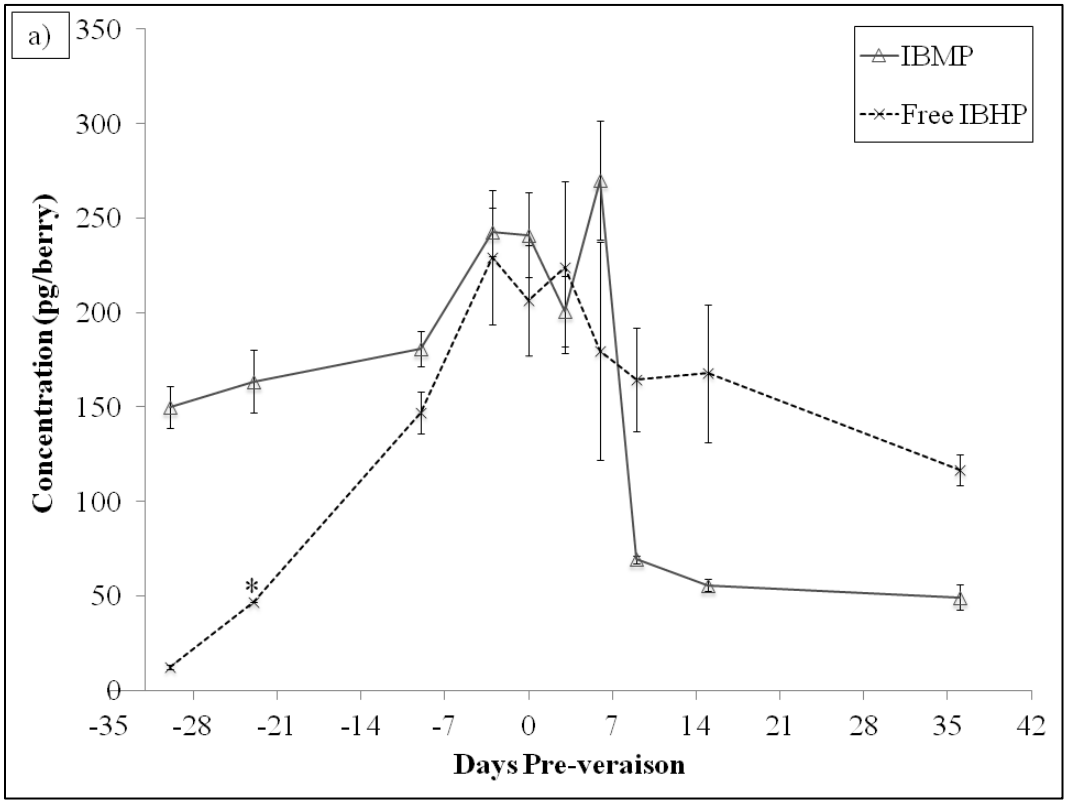
In summary, the use of mixed mode cation exchange SPE followed by silylation and one dimensional GC-TOF-MS for quantification can achieve LOD of 20 ng/L for 100 mL sample sizes. This is a considerable improvement over the earlier method described by our group which required 1000 mL sample sizes and GCxGC-TOF-MS for quantification to achieve a similar LOD [12].

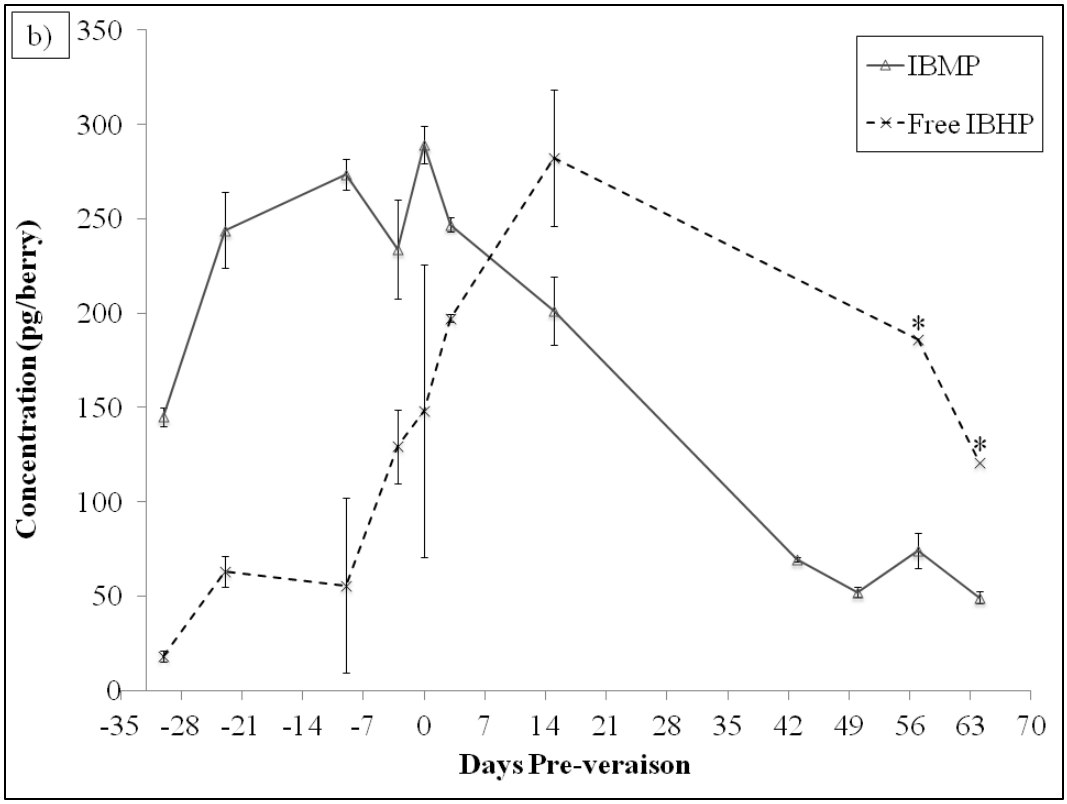
Time Course Studies of IBHP and IBMP

IBHP in berries was measured during the 2010 growing season in both California Merlot and New York Cabernet franc. For the Cabernet franc, two different clones were studied. Time course profiles for pg IBHP/berry vs. time are shown in Figure 9, and all three trials showed similar profiles. In all trials, IBHP was detectable at the earliest sampling point (4 weeks pre-veraison, or approximately 3 weeks post-bloom), increased to a maximum on a per-berry basis at 1-2 weeks post-veraison, and then decreased during ripening. For example, CF4 (Figure 5a) had 12 pg IBHP/berry (21 pg/g) at 30 days pre-veraison, which increased to 229 pg/berry (254 pg/g) by 3 days pre-veraison. IBHP did not change significantly until 36 days post-veraison where it decreased to116 pg/berry (77 pg/g). Similar patterns were observed for the other trials. Interestingly, IBHP in the California Merlot (Figure 5c) reached a higher maximum value, peaking at 477 pg/berry (835 pg/g) at 8 days post-veraison, or nearly double the maximum IBHP observed on both a per-berry and concentration basis in the New York Cabernet franc samples. However, the late season decrease in IBHP in the California Merlot samples occurred more

rapidly than in the New York Cabernet franc, with a 75% decrease observed between 8 days and 22 days post-veraison. Due to the limited number of trials included in this study, it is not clear why IBHP degradation was more rapid in the California Merlot.

Although this is the first report to perform a detailed time course study on IBHP, previous reports have measured IBHP in Cabernet franc with only 11 days pre-veraison , 28 days post-veraison and harvest (59 days post-veraison) time points [12]. The previous observed IBHP concentrations were much lower for similar pre- and post-veraison time points than were determined in this study, although measurements taken at harvest were much higher. Whether this is due to measurement differences or clone or seasonal differences it is hard to tell due to the lack of available data points.





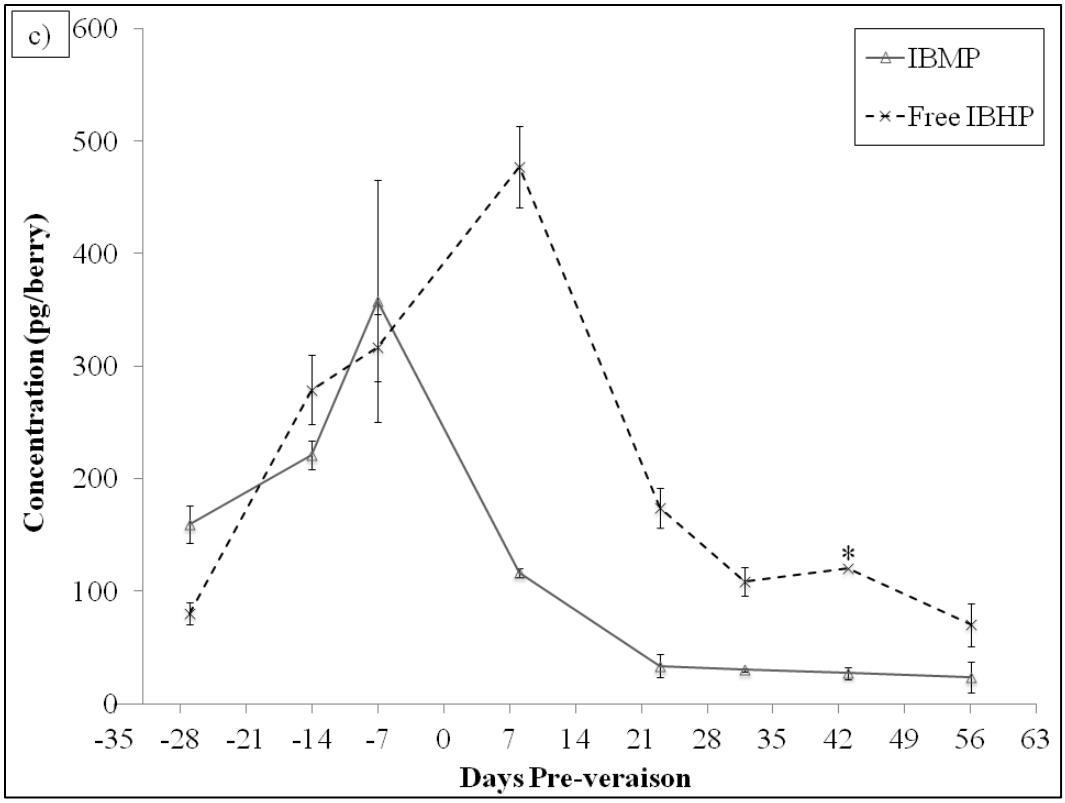


Figure 9. IBMP and free IBHP concentrations during ripening over the 2010 growing season for (a) CF4 (NY), (b) CF1 (NY) and (c) Merlot (CA). The error bars show the standard deviation of the duplicates. (*) Indicates no replicates were done.

The concentrations of IBHP for CF1 and Merlot peaked at least 2 weeks after IBMP reached a maximum (Figure 9). Similar to previous reports [6, 13], IBMP increased early in the season until 0-2 weeks pre-veraison, after which IBMP then slowly decrease until harvest. The IBMP concentrations of CF4 were very different than expected, with a peak IBMP concentration occurring 7 days after veraison. This could potentially be due to natural variation within the berries, as without this point the time course follows the general IBMP trend. CF4 and CF1 IBMP peaked at a level of 269 pg/berry (198 pg/g) and 289 pg/berry (340 pg/g), at 7 and 0 days post-veraison, respectively. IBMP concentrations had declined to 49 pg/berry (33 pg/g) and 49 pg/berry (25 pg/g) by 35 and 64 days post veraison, respectively. Merlot peaked at 357 pg/berry (631 pg/g) 7 days pre-veraison then decreased to a concentration of 24 pg/berry (20 pg/g) at 56 days post veraison. Merlot had the highest IBMP concentration as well as the highest peak of free IBHP, while CF4 had both the lowest peak IBHP and peak IBMP concentrations, though similar to CF1. This could suggest a trend between peak free IBHP and peak free IBMP, though this cannot be confirmed without more data.

The observation that IBHP continues to increase for 1-2 weeks after veraison while IBMP begins to decline during the same time period is of note. Dunlevy, et al observed a decrease in *VvOMT1* expression between 8 and 10 weeks post-flowering, correlating with a decrease in IBMP during the same time [21]. Thus, although IBHP concentrations are still increasing, IBHP is no longer methylated to IBMP by *VvOMT1* after 1-2 weeks pre-veraison.

Previous reports had observed similar concentrations of IBHP in both IBMP accumulating (Cabernet franc) and non-accumulating (Riesling, Pinot noir) cultivars [12]. This suggests that differences in IBMP among cultivars may be explained by differences in *VvOMT1* activity, a statement supported by computational studies on *VvOMT1* from Pinot noir and Cabernet

Sauvignon. In our current work, we observed the highest concentration of IBHP in grapes from the trial with the highest IBMP accumulation – California Merlot. This suggests that differences in IBMP accumulation may also be governed at least in part by differences in IBHP accumulation. However, *VvOMT1* activity was not measured in our current study, so it is not possible to confirm this.

IBHP in Grapes Following Acid Hydrolysis

We had previously observed an increase of IBHP in juice samples following acidification and heating of samples [12], potentially because a fraction of IBHP exists in a 'bound' glycosylated form as has been observe in rat urine following IBMP ingestion. Assuming this glycosylated IBHP pool is stable, we had reasoned that this bound IBHP pool could be used as a proxy for maximum IBMP concentrations [12].

Table 3. Total, free and bound IBHP (pg/berry) in CF4. Different letters indicate a statistically significant difference at $p < 0.05$.

Days Post-Veraison	Total IBHP	Free IBHP	Bound IBHP [Total IBHP – Free IBHP]
-3	1179[a]	229[b]	950[a]
0	1220[ab]	206[b]	1013[ab]
9	741[a]	165[b]	576[ab]
14	558[ab]	168[b]	391[ab]

Table 4. Total, free and bound IBHP (pg/berry) in CF1. Different letters indicate a statistically significant difference at $p < 0.05$.

Days Post-Veraison	Total IBHP	Free IBHP	Bound IBHP [Total IBHP – Free IBHP]
57	210[a]	186[a]	24[a]

To test this hypothesis, acid hydrolysis was performed on CF4 and CF1 samples prior to IBHP measurement (Table 3 and Table 4). Total IBHP content (acid-releasable and free) was considerably higher than free IBHP in CF4, and reached a maximum of 1179 pg/berry (1304 pg/g) at 3 days pre-veraison. Total IBHP in CF4 decreased more rapidly than free IBHP after veraison to a level of 741 pg/berry (574 pg/g) by 9 days post veraison. The total IBHP sample from CF1 showed only 210 pg/berry (92 pg/g) and 186 pg/berry (82 pg/g) free IBHP by 57 days post-veraison. As the total IBHP at this point was not significantly different from the free IBHP, this equates to a concentration of bound IBHP not distinguishable from zero. This suggests that the concentration of bound IBHP decreases to below the limit of detection after veraison. It is of note that when IBHP was measured in bell peppers, there was no bound form of IBHP [12]; however as the bound form IBHP was only measured in ripe bell pepper it could be possible for no bound IBHP to remain by ripeness, if the same degradation pattern exists.

This highlights the question of the synthesis and degradation pathway of IBMP and IBHP. The pathway of IBHP could potentially be explained due to the structural similarity to the aglycone flavanol, quercetin. Both IBHP and quercetin have 3' hydroxyl active groups on a planar, heterocyclic 6 member carbon ring (Figure 10). Quercetin is a natural occurring phenol that is thought to act as an antioxidant and UV-protectant in grapes [38]. The enzymes VvOMT1 and VvOMT2 both have higher than 100x more activity against quercetin than they do against IBHP [21] and it appears to be this promiscuity in the OMTs that allow for the side activity that causes synthesis of IBMP. IBMP demethylation is proposed to be through a 3-*o*-demethylase [12, 22] and though there are several potential enzymatic families that are capable of demethylation, such as cytochrome P450s, FAD dependent oxidases and dioxygenases, an enzyme capable of 3-*o*-demethylation of a planar heterocylic ring in plants has yet to be

characterized [31]. It seems as though there are different degradation patterns of IBHP, which could plateau, decrease rapidly, or decrease slowly. This suggests that the mechanisms that govern IBHP concentrations are separate from those that control IBMP metabolism and that IBMP is a side activity from the IBHP pathway.

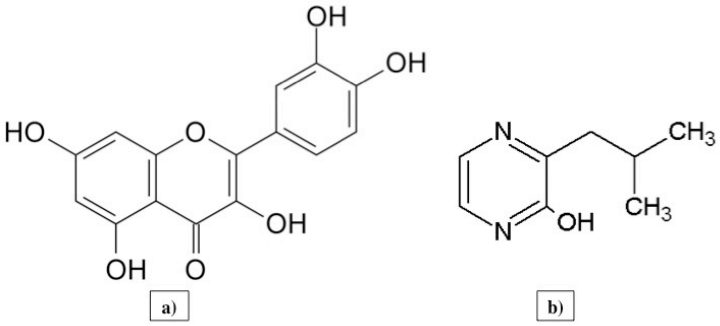


Figure 10. The similar structures of a) Quercetin and b) IBHP.

The acid labile IBHP, or bound IBHP, was hypothesized to be the glycosylated form of IBHP [12]. It is possible that due to the similarity of quercetin and IBHP as a substrate that the same enzyme that glycosylases quercetin to quercetin-3-glucose could glycosylate IBHP into IBHP-3-glucose. Glycosylation is a common reaction catalyzed by several glycosyltransferase enzymes in plants shown to create stable, non-toxic products [32]. UFGT (UDP glucose-flavonoid 3-*o*-glycosyl transferase) coincides with the accumulation of anthrocyanins in the exocarp of the berry at veraison, and so could also explain the presence of bound IBHP at veraison [33]. UGTs (UDP glycosyl transferases) are quite region selective as seen with quercetin as it has to block several other active sites while glycosylating one specific site [34, 35]. However, UGTs are promiscuous against several types of small lipophilic molecules with common features (including hormones, endogenous metabolites and xenobiotics) [36] which lends credence to the hypothesis that IBHP and quercetin are glycosylated by a similar or the same enzyme. One

argument against the glycosylation of IBHP is due to the degradation or transformation of the bound form of IBHP – as glycosylated compounds are incredibly stable throughout the growth of plants, it seems unlikely that the glucosyl bond is broken; however, the transformation could be explained with a change in the IBHP ring or side group.

Evaluating Ability of Yeast to Methylate IBHP to IBMP - Winemaking and Resting Cell Experiments

Several *O*-methyltransferases have been identified for *Saccharomyces cerevisiae* [37], although it is not known if these *OMTs* can methylate IBHP to IBMP as has been demonstrated for *VvOMT*. Since free IBHP concentrations were nearly 100 pg/g at harvest at one site in this study, and over 200 pg/g in a previous study, even modest activity towards IBHP by yeast during fermentation could result in a meaningful change to IBMP in the finished wine.

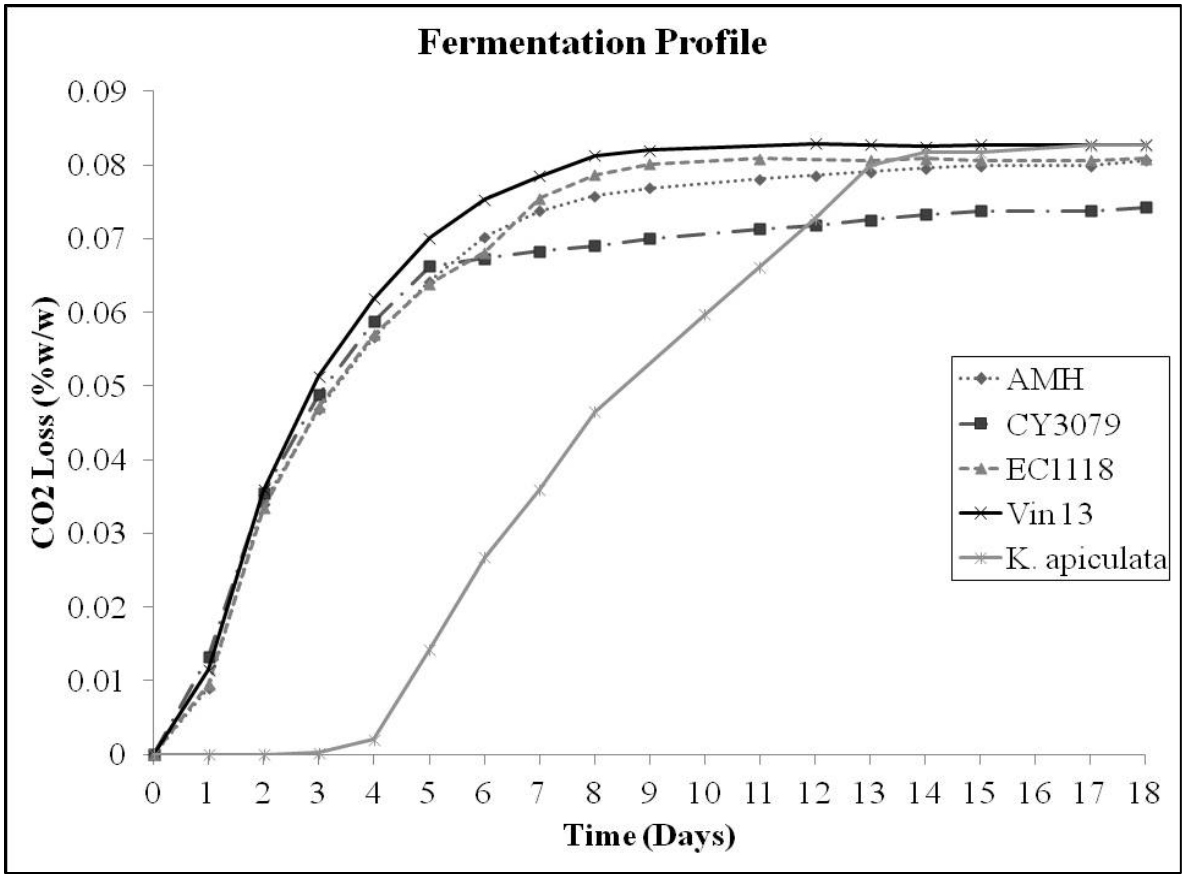


Figure 11. Fermentation profile of five yeast strains in pasteurized Pinot gris grape juice at 25˚C in the presence of 1 µg/g IBHP.

To determine wine yeasts exhibited could transform IBHP to IBMP, small scale fermentations on spiked juices were carried out with 4 commercial *S. cerevisiae* wine yeast strains, AMH, CY3079, EC1118 and VIN13, and one non-*Saccharomyces* strain (*K. apiculata*) were carried out in triplicate. Pinot gris grape juice was chosen as the fermentation medium because no detectable IBMP was present in the juice (<2 ng/L). 1 µg/L IBHP was added at the time of inoculation. Fermentation kinetics of the system were monitored by observing changes in the mass of the fermentation containers and loss of CO_2 (Figure 11). All four *Saccharomyces* strains followed a similar fermentation pattern, and were finished fermenting by 10 days post inoculation. CY3079 did not ferment to dryness even though no further fermentation activity was taking place. *K. apiculata* had a much longer lag phase than the other strains, and did not rapidly

ferment until 4 days post inoculation, but then proceeded to ferment the juice to dryness by 15 days post inoculation. No significant difference in kinetics was observed in the samples with added IBHP. Throughout fermentation, IBMP measurements were taken at 2-4 days intervals. The LOD for IBMP in wine using the 1-D GC-TOF-MS method was ca. 5 pg/mL, and no IBMP was detected at any time during fermentation. Thus, if IBMP was being formed by methylation during fermentation, it was less than 0.5% of the available IBHP pool.

A resting cell experiment using the commercial yeast strain EC1118 (*S. cerevisiae*) was also used to evaluate the ability of wine yeast to methylate IBHP. Two trials were conducted in duplicate: the first was done using 1 μg/g IBHP as a substrate to determine if the yeast exhibited *OMT* activity leading to the production of IBMP, and the second was performed using 1 μg/g IBHP and 1 μg/g IBMP to determine if IBMP exhibited a positive control on any *OMT* gene expression. Over the course of 2 h, no detectable change in IBMP concentration was observed in either trial. As further confirmation, we did not detect any characteristic IBMP aroma in the 1 μg/g IBHP trial.

Conclusions

In conclusion, we have monitored both free and acid-releasable IBHP in grapes during the growing season using an improved methodology capable of 20 ng/L detection limits with a 100 mL sample size. Free IBHP concentrations peak at least 2 weeks after the maximum IBMP concentration is reached and then decline during ripening. Total IBHP is nearly 6-fold higher than free IBHP at veraison, but decreases post-veraison, indicating that it cannot be used as a proxy for maximum IBMP. Because the highest free IBHP concentrations were observed in grapes with the highest IBMP concentrations, it is possible that maximum IBMP accumulation is

influenced by IBHP accumulation. Future studies to determine if IBHP accumulation is more important than differences in *VvOMT* activity in explaining differences in IBMP accumulation seem justified, especially since harvest IBMP is often well correlated with maximum pre-veraison IBMP. Finally, wine yeasts do not appear to possess the ability to methylate IBHP to IBMP during fermentation. Thus, IBHP in harvested grapes does not present a potential source of IBMP in wines. This conclusion is supported by the strong correlation between IBMP concentrations in grapes at harvest and in the resulting wines [28].

WORKS CITED

1. Buttery, R.G., Seifer, R.M., Guadagni, D.G., Ling, L.C., *Characterization of Some Volatile Constituents of Bell Peppers.* J. Agr. Food Chem., 1969. **17**(6): p. 1322-1327.

2. Bayonove, C.R., R. Cordonnier, and P. Dubois, *Etude d'une fractions caractéristique de arôme du raison de la variété Cabernet-Sauvignon; mise en evidence de la 2-méthoxy-3-isobutylpyrazine.* C. R. Acad. Sci. Paris. Ser. D, 1975. **281**: p. 75-78.

3. Murray, K.E. and F.B. Whitfield, *The Occurrence of 3-Alkyl-2-methoxypyrazines in Raw Vegetables.* J. Sci. Fd. Agric., 1975. **26**: p. 973-986.

4. Seifert, R.M., Buttery, R.G., Guadagni, D.G., Black, D.R., Harris, J.G., *Synthesis of Some 2-Methoxy-3Alkylpyrazines with Strong Bell Pepper-Like Odors.* J. Agr. Food Chem., 1970. **18**(2): p. 246-249.

5. Allen, M.S., et al., *Contribution of Methoxypyrazines to Sauvignon blanc Wine Aroma.* Am. J. Enol. Vitic., 1991. **42**(2): p. 109-112.

6. Roujou de Boubée, D., C. Van Leeuwen, and D. Dubourdieu, *Organoleptic Impact of 2-Methyoxy-3-isobutylpyrazine on Red Bordeaux and Loire Wines. Effect of Environmental Conditions on Concentrations in Grapes during Ripening.* J. Agr. Food Chem., 2000. **48**: p. 4830-4834.

7. Preston, L.D., et al., *Defining vegetal aromas in Cabernet Sauvignon using sensory and chemical evaluations.* Am. J. Enol. Vitic., 2008. **59**: p. 137-145.

8. Ebeler, S.E. and J.H. Thorngate, *Wine Chemistry and Flavor: Looking into the Crystal Glass.* J. Agr. Food Chem., 2009. **57**: p. 8098-8108.

9. Marais, J., *Sauvignon blanc Cultivar Aroma - A Review.* S. Afr. J. Enol. Vitic., 1994. **15**(2): p. 41-45.

10. Bogart, K. and L. Bisson, *Persistence of vegetal characters in winegrapes and wine.* Practical Vineyard and Winery, 2006. **86**: p. 13-20.

11. Allen, M.S., M.J. Lacey, and S. Boyd, *Determination of Methoxypyrazines in Red Wines by Stable Isotope Dilution Gas Chromatography-Mass Spectrometry.* J. Agric. Food Chem, 1994. **42**(8): p. 1734-1738.

12. Ryona, I., S. Leclerc, and G.L. Sacks, *Correlation of 3-Isobutyl-2-methoxypyrazine to 3-Isobutyl-2-hydroxypyrazine during Maturation of Bell Pepper (Capsicum annum) and Wine Grapes (Vitis vinifera).* J. Agr. Food Chem., 2010. **58**: p. 9723-9730.

13. Ryona, I., et al., *Effects of Cluster Light Exposure on 3-Isobutyl-2-methoxypyrazine Accumlulation and Degradation Patterns in Red Wine Grapes (Vitis vinifera L. Cv. Cabernet Franc).* Am. J. Enol. Vitic., 2008. **56**: p. 10838-10846.

14. Sala, C., et al., *Influence of Vine Training and Sunlight Exposure on the 3-Alkyl-2-methoxypyrazines Content in Musts and Wines from the Vitis vinifera Variety Cabernet Sauvignon.* J. Agr. Food Chem., 2004. **52**: p. 3492-3497.

15. Sala, C., J. Guasch, and F. Zamora, *Factors Affecting the Presence of 3-Alkyl-2-methoxypyrazines in Grapes and Wines. A Review.* 2004, Universitat Rovira i Virgili. p. pp 53-58.

16. Scheiner, J., et al., *Modeling Impacts of Viticultural and Environmental Factors on 3-Isobutyl-2-methoxypyrazine in Cabernet franc Grapes.* Am. J. Enol. Vitic., 2012. **63**(1): p. 94-105.

17. Scheiner, J.J., G.L. Sacks, and J.E. Vanden Heuvel, *How Viticultural Factors Affect Methoxypyrazines*, in *Wines & Vines*. 2009.

18. Rizzi, G.P., *Formation of Methoxypyrazines in Reactions of 2(1H)-Pyrazinones with naturally Occuring Methylating Agents.* J. Agric. Food Chem, 1990. **38**: p. 1941-1944.

19. Hashizume, K., et al., *Purification and Characterization of a O-Methyltransferase Cabaple of methylating 2-Hydrozy-3-alkylpyrazine from Vitis viniferai L. (cv. Cabernet Sauvignon).* Biosci. Biotech. Biochem., 2001. **65**(10): p. 2213-2219.

20. Hashizume, K., et al., *S-Adenosyl-L-methionine-dependent O-Methylation of 2-Hydroxy-3-alkylpyrazine in Wine Grapes: A Putative Final step of Methoxypyraxzine Biosynthesis.* Biosci. Biotech. Biochem., 2001. **65**(4): p. 795-801.

21. Dunlevy, J.D., et al., *Two O-methyltransferases Involved in the Biosynthesis of Methoxypyrazines: Grape-Derived Aroma Compounds Important to Wine Flavor.* Plant Mol. Biol., 2010. **74**: p. 77-89.

22. Hawksworth, G. and R.R. Scheline, *Metabolism in the Rat of Some Pyrazine Derivatives having Flavour Importance in Foods.* Xenobiotica, 1975. **5**(7): p. 389-399.

23. Kotseridis, Y., R. Baumes, and G.K. Skouroumounis, *Synthesis of Labelled $^{2}[H_4]$-β-damascenone, $^{2}[H_2]$-2-methyoxy-3-isobutylpyrazine, $^{2}[H_2]$-α-ionone, and $^{2}[H_2]$-β-ionone, for Quantification in Grapes, Juice and Wines.* J. Chromatogr., 1998. **824**: p. 71-78.

24. Bertz, S.H., *Convenient Laboratory Preparation of Glyoxal-d_2 and 2,4,6,8-Tetrakis(methoxycaronyl)bicylclo[3.2.0]octane-3,7-dione-1,5-d_2.* J. Org. Chem., 1981. **46**: p. 4088-4090.

25. Gerritsma, D.A., et al., *Preperation of Labelled 2-Methoxy-3-alkylpyrazines: Synthesis and Characterization of Deuterated 2-Methyoxy-3-isopropylyrazine and 2-Methoxy-3-isobutylpyrazine.* J. Labelled Compd. Radiopharm., 2003. **4+**: p. 243-253.

26. Sweeley, C.C., et al., *Gas-Liquid Chromatography of Trimethyl Derivatives of Sugars and Related Substances.* J. Am. Chem. Soc., 1963. **85**: p. 2497-2507.

27. Mac Berthoeux, P. and L.C. Brown, *Statistics for Environmental Engineers*. 2 ed. 2002, Boca Raton, FL: CRC.

28. Ryona, I., B.S. Pan, and G.L. Sacks, *Rapid Measurement of 3-Alkyl-2-methoxypyrazine Content of Winegrapes to Predict Levels in Resultant Wines.* J. Agr. Food Chem., 2009. **57**: p. 8250-8257.

29. Fia, G., G. Giovani, and I. Rosi, *Study of β-Glucoside Production by Wine-Related Yeasts During Alcoholic Fermentation. A New Rapid Fluorimetric Method to Determine Enzymatic Activity.* J. Appl. Microbiol., 2005. **99**.

30. López, R., et al., *Development of a Mixed-Mode Solid Phase Extraction Method and Further Gas Chromatography Mass Spectrometry for the Analysis of 3-Alkyl-2-methoxypyrazines in Wine.* J. of Chrom. A., 2011. **1218**: p. 842-848.

31. Hagel, J.M. and P.J. Facchini, *Biochemistry and occurrence of O-demethylation in plant metabolism.* Front. in Phys., 2010. **1**: p. 1-7.

32. Bowles, D.J., et al., *Glycosyltransferases: managers of small molecules.* Current Opinion in Plant Biol., 2005. **8**: p. 254-263.

33. Ford, C.M., P.K. Boss, and P.J. Høj, *Cloning and Characterization of Vitis vinifera UDP-Glucose: Flavonoid 3-O-Glucosyltransferase, a Homologue of the Enzyme Encoded by the Maize Bronze-1 Locus That May Primarily Serve to Glucosylate Anthocyanidins in Vivo.* J. of Bio. Chem., 1998. **15**: p. 9224-9233.

34. Lim, E., et al., *Evolution of substrate recognition across a multigene family of glycosyltransferases in Arabidopsis.* Glycobiol., 2003. **13**: p. 139-145.

35. Lim, E., et al., *Arabidopsis Glycosyltransferases as Biocatalysts in Fermentation for Regioselective Synthesis of Diverse Quercetin Glucosides.* Biotech. and Bioeng., 2004. **87**: p. 623-631.

36. Lim, E. and D.J. Bowles, *A class of plant glycosyltransferases involved in cellular homeostasis.* EMBO J., 2004. **23**: p. 2915-2922.

37. Niewmierzycka, A. and S. Clarke, *S-Adenosylmethionine-dependent Methylation in Saccharomyces cerevisiae.* J. of Bio. Chem., 1999. **247**: p. 814-824.

38. Price, S.F., Breen, P.J., Valladao, M., and B.T. Watson, *Cluster Sun Exposure in Quercetin in Pinot noir Grapes and Wine*. Am. J. Enol., Vitic., 1995. **46**: p. 187-194.