JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Behavior of 3-Isobutyl-2-hydroxypyrazine (IBHP), a Key Intermediate in 3-Isobutyl-2-methoxypyrazine (IBMP) Metabolism, in Ripening Wine Grapes

Sarah A. Harris, Imelda Ryona, and Gavin L. Sacks*

Department of Food Science, Cornell University, 630 West North Street, Geneva, New York 14456, United States

Supporting Information

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 its behavior during the growing season is not well understood. First, an improved method for IBHP quantification was developed. A deuterated IBHP standard was added to samples prior to isolation by mixed-mode cation exchange solid phase extraction. Extracts were silylated prior to quantification by GC-MS. A limit of detection of ca. 20 ng/L could be achieved for a 100 mL juice sample. This method was used to quantify IBHP during the 2010 growing season in berries of two clones of Cabernet franc in the Finger Lakes region of New York and of Merlot grown in the California Central Valley. For all three sources, IBHP was detectable at the earliest sampling point, and its concentration per berry increased to a maximum around veraison, 208−477 pg/berry. On a per berry basis, IBHP peaked and began to decline 1−2 weeks after IBMP, indicating that previous studies that sampled preveraison fruit have missed the true maximum value of IBHP. The highest per berry concentration of IBHP observed was in the California Merlot. However, after veraison, IBHP declined more rapidly in the California Merlot than in the New York Cabernet franc, such that the Merlot had the lowest IBHP concentration at harvest. Thus, IBHP at harvest cannot be used as a proxy for IBMP at veraison as was previously suggested.

KEYWORDS: *grape maturation, grape aroma, wine aroma, pyrazines*

INTRODUCTION

The 3-alkyl-2-methoxypyrazines (MPs), including 3-isopropyl-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 ng/L in water or wine,[4−6] and their concentrations in vegetative tissue and unripe fruits can exceed 1000 ng/kg.[3]

The MPs, and particularly IBMP, are known to play an important role in the flavor of some wines,[5,7−10] including varietals such as Sauvignon blanc, Cabernet Sauvignon, and Cabernet franc.[5,10,11] Whereas 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.[12−16] However, interpretation of these empirical results is often challenging. For example, several authors have observed that preveraison cluster shading in the vineyard results in increased accumulation of IBMP,[15,17,18] but a biochemical explanation for this phenomenon is not available.

Interpretation of these viticultural studies should be facilitated by understanding the behavior of metabolic intermediates of MPs. MP biosynthesis in plants is hypothesized to begin with the condensation of NH_3 with an amino acid (e.g., leucine, valine) and glyoxal to form a 3-alkyl-2(1*H*)-pyrazin-2-one and its tautomer, 3-alkyl-2-hydroxypyrazine (HP).[3,4] Subsequently, HPs are thought to be *O*-methylated to form MPs.[3,19] An *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferase (OMT) capable of methylating HPs into MPs has been identified and purified.[20,21] 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 preveraison, corresponding with the time of maximum IBMP accumulation within grape berries.[22] The degradation pathway of IBMP in plants is not as well studied. A recent paper has suggested that IBMP may be demethylated to re-form IBHP and then partially glycosylated,[23] similar to metabolism of IBMP observed in rats.[24]

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 the 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,[21,23] and both consider only a

Received: July 10, 2012
Revised: October 17, 2012
Accepted: November 19, 2012
Published: November 19, 2012

ACS Publications © 2012 American Chemical Society

Table 1. Experimental Vineyard Sites Used in This Work

sample	location	cultivar	veraison date	training system and spacing	year est	soil type
CF4	Cayuga Lake, NY	Cabernet franc Cl. 4	Aug 15	VSP, 2.7 m × 1.8 m	2008	gravelly loam
CF1	Cayuga Lake AVA, NY	Cabernet franc Cl. 1	Aug 15	VSP, 2.7 m × 1.8 m	2008	gravelly loam
Merlot	Central Valley, CA	Merlot	July 15	sprawl, 3.0 m × 1.8 m	1996	sandy loam

limited number of time points. In part, this may reflect analytical difficulties, because IBHP's amphiphilic nature, low volatility, and low concentration make its quantification difficult.

In this study, we describe an improved method for IBHP quantification. IBHP is extracted by mixed-mode solvent phase extraction (SPE) in the presence of a deuterated standard, silylated, and quantified by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). We then present data on the correlation of IBHP and IBMP concentrations during the growing season across multiple sites.

MATERIALS AND METHODS

Chemical Reagents and Standards. 3-Isobutyl-2-hydroxypyrazine was purchased from Manchester Organics Ltd. (97%, Sutton Weaver, UK). Synthesis of $^2[H_2]$-3-isobutyl-2-hydroxypyrazine is described in a subsequent paragraph. Sodium chloride (NaCl), potassium carbonate, sodium hydroxide (NaOH), D-glucose, pyridine (99%), hexamethyldisilane (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, USA); ethylenediaminetetraacetic acid (EDTA), citric acid, ascorbic acid, sodium chloride (NaCl), ethanol, and trimethylsilyl chloride (TMCS) were purchased from Acros Organics NV (Geel, Belgium); ethyl acetate and acetonitrile were obtained from VWR International (West Chester, PA, USA); and dichloromethane, ammonium hydroxide, calcium chloride, tartaric acid, hydrochloric acid (37%), methanol, dichloromethane, magnesium sulfate, and D-fructose were purchased from Fisher Chemical (Fair Lawn, NJ, USA). YPD agar and YPD broth were purchased from B. D. Difco (Franklin Lakes, NJ, USA). Deionized, distilled water was obtained from a Milli-Q purification system Millipore (Billerica, MA, USA).

$^2[H_2]$-3-Isobutyl-2-hydroxypyrazine ($^2[H_2]$-IBHP) Synthesis. $^2[H_2]$-IBHP synthesis was adapted from previous methods.[25,26] 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 h to yield a white crystalline solid, $^2[H_2]$-glyoxal bis(sodium bisulfite)-*O*-$^2[H_2]$,[25] which was used in the next step without further purification. In a separate round-bottom flask, leucinamide hydrochloride (0.17 g) was dissolved in 2 mL of methanol and cooled to −35 °C. The $^2[H_2]$-glyoxal bis(sodium bisulfite)-*O*-$^2[H_2]$ slurry (0.17 g) was added and stirred vigorously. Aqueous NaOH (12 M, 200 μL) was added dropwise over the course of 20 min. The solution was warmed to room temperature and stirred continuously for 2 h. The mixture was cooled to 0 °C and acidified with 200 μL of 12 M HCl followed by the 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 $^2[H_2]$-IBHP was extracted from the aqueous layer with 3 × 5 mL aliquots of dichloromethane. The EI-MS and ^{1}H NMR data were similar to data from Gerritsma et al.[26] Following silylation, the following GC-MS spectrum was observed [*m*/*z* (RI%)]: 153 (19), 169 (88), 184 (100), 211 (28), consistent with a 2 amu shift as compared to the IBHP spectra presented in Hawksworth et al.[24]

Fruit Samples for Time Course Studies of IBMP and IBHP. 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 1. For sampling, 1 kg of grape clusters was selected at random from throughout a vineyard block and frozen at −4 °C prior to analysis. Samples were taken at either weekly or biweekly intervals from fruit set until harvest when possible, although some samples could not be collected at the expected interval because of unplanned pesticide spraying. For recovery experiments, frozen Cabernet franc clusters harvested in 2008 from experimental vineyards in Geneva, NY, were used.

Grape Juice Preparation. Frozen grapes (400 g) were thawed, manually destemmed, and homogenized with a Waring blender (model 5011, Torrington, CT, USA) at low speed for 1 min in the presence of 50 mg/kg ascorbic acid to prevent browning and potential oxidative losses. 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,[23] the insoluble solid material was discarded. An aliquot of juice was separated for measurements of TA and soluble solids. The filtered juice was loaded into either 250 or 500 mL Nalgene polycarbonate centrifuge bottles (VWR International) and centrifuged at 9000 rpm for 30 min at 4 °C (Sorvall RC6+ centrifuge, Thermo Scientific, Waltham, MA, USA). 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 and Berry Weight Measurements. Soluble solids were measured on extracted juice by a digital refractometer (Leica Auto ABBE; AO Scientific Instruments, Buffalo, NY, USA). The titratable acidity (TA) was measured with an automatic titrator (Titrino Plus 848 Doser, 869 Autosampler, Methrohm USA, Riverview, FL, USA) using an end point of pH 8.2. Subsamples of 100 berries were weighed to determine mean berry weight.

IBHP Extraction via Cation-Exchange SPE. SPE of IBHP from grape juice was performed on a Varian 24 cartridge positive pressure manifold (Palo Alto, CA, USA), using 6 mL cartridges packed with 200 mg of Bond Elut Plexa PCX sorbent (Agilent, Santa Clara, CA). Cartridges were conditioned with 5 mL of dichloromethane, 10 mL of methanol, and 20 mL of water prior to sample loading. 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. Each sample was split into two 50 mL subsamples and extracted in parallel on two identically conditioned cartridges to expedite sample processing. The loaded cartridges were washed with 6 mL of 5% v/v methanol solution adjusted to pH 2 with HCl, and the cartridge was dried with N_2 (25 psi) for 30 min. 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.[24] and Sweeley et al.[27] The dried extract was reconstituted in 0.5 mL of dry pyridine, after which 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.[24] The solution was heated for 20 min at 65 °C, cooled, and then analyzed by GC-TOF-MS without further extraction of the reaction mixture. If sample extracts were not to be analyzed immediately, the extracts were kept in a −20 °C freezer and analyzed within 6 h of removal from the freezer to minimize hydrolysis of the silylated derivative.

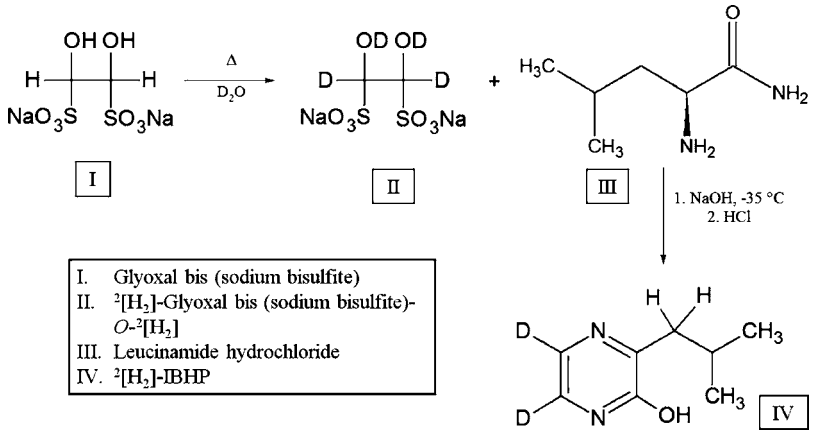

Figure 1. Synthesis scheme for $^2[H_2]$-IBHP.

Quantification of Derivatized IBHP Extracts by GC-TOF-MS. The derivatized IBHP was quantified by GC-TOF-MS (Pegasus, LECO Corp., St. Joseph, MI, USA). The GC system was a comprehensive 2-D GC (GC×GC), 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 × 0.25 mm × 0.25 μm, Agilent) coupled to a DB-17 (2 m × 0.1 mm × 0.2 μm, Agilent) via an inert glass press-tight connector. Three microliters was 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 an 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 LECO ChromaTOF software. The qualifier ions for IBHP were m/z 151, 167, and 209, and the quantifier ion was m/z 182. The qualifier ions for $^2[H_2]$-IBHP were m/z 153, 169, and 211, and the quantifier ion was m/z 184. Quantification was performed with respect to appropriate calibration curves, described below. IBHP concentrations (pg/g) were converted to units of picograms per berry by multiplying the concentration by the mean berry weight.

Calibration Curve, Limits of Detection, and Recovery for IBHP. Calibration curves for IBHP were prepared in 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 ratio of 3:1 and was estimated from calibration curves using Pallesen's method.[28] To determine if model juice calibration curves were appropriate for real juice samples, a recovery experiment was performed by the method of standard addition. One hundred milliliters of Cabernet franc juice was either spiked with 50 ng of IBHP or left unspiked. The spiked and unspiked samples were extracted by using the SPE protocol described above. Triplicates of both spiked juice and spiked reference samples were analyzed. Recovery was calculated as the ratio of the observed concentration to the expected concentration of IBHP in the spiked samples.

Analysis of Total IBHP by Acid Hydrolysis. Time course samples from Cabernet franc clone 4 were evaluated for acid-releasable IBHP. Juice samples were acidified to pH 2 by the addition of HCl, and $[^2H]_2$-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 grape samples was quantified by GC×GC-TOF-MS using a method described elsewhere.[29] IBMP concentrations (pg/g) were converted to units of picograms per berry by multiplying the concentration by the mean berry weight.

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

RESULTS AND DISCUSSION

Synthesis of $^2[H_2]$-IBHP Internal Standard. The protocol used for synthesizing the $^2[H_2]$-IBHP internal standard (Figure 1) was adapted from an earlier approach by Gerritsma et al.[26] in which $^2[H_2]$-glyoxal was condensed with the HCl salt of leucimanide (**III**) to generate $^2[H_2]$-IBHP (**IV**). In the earlier paper, the bisulfate salt of $^2[H_2]$-glyoxal was synthesized by reduction of diethyl oxalate with lithium aluminum deuteride. In our current work, the prepared $^2[H_2]$-glyoxal bis(sodium bisulfite)-O-$^2[H_2]$ (**II**) was synthesized by D/H exchange of unlabeled glyoxal bis(sodium bisulfite) (**I**) as previously described.[25] The reaction of **II** and **III** then took place under the previously recommended conditions.[26] The EI-MS and NMR data for $^2[H_2]$-IBHP were consistent with the earlier paper of Gerritsma et al.,[26] with the EI-MS of the deuterated standard showing a consistent 2 amu shift as compared to the unlabeled standard. No undeuterated IBHP was detected by either MS or NMR, but a small amount of monodeuterated $[H_2]$-IBHP was also observed and estimated to be 12% of the $^2[H_2]$-IBHP signal by NMR. This was presumably due to incomplete labeling of the glyoxal, but its presence did not affect the use of the synthesized $^2[H_2]$-IBHP as an internal standard.

SPE Method. An optimized protocol was developed for isolating the basic IBHP analyte by mixed-mode cation-exchange 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 study, as the use of H_3PO_4 resulted in a sizable trisilylphosphate interference following derivatization (data not shown).

Previously, Lopez et al. had recommended 30% methanol as a wash for mixed-mode SPE isolation of IBMP.[30] However, IBHP is expected to be more polar than IBMP, and in our own work, we observed a 30−50% decrease in IBHP signal with only 20% methanol in the wash step without a corresponding improvement in interference removal (data not shown). For this reason, we used 6 mL of 5% methanol/water (v/v), pH 2, as the wash solvent. The optimal eluting solvent was 20% (v/v) dichloromethane in ethyl acetate made basic by the addition of 2% NH_4OH. Slightly higher recoveries could be achieved with

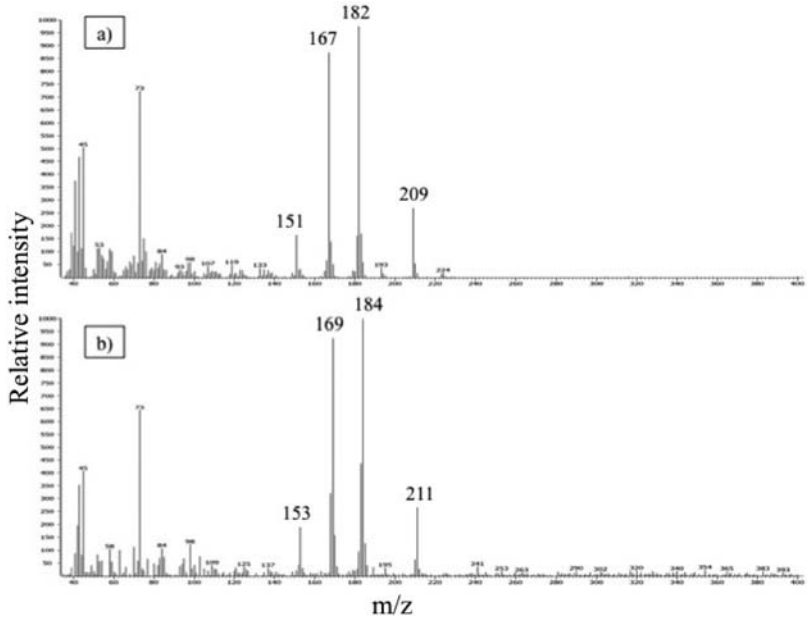

Figure 2. GC-TOF-MS mass spectra displaying qualifying ions of the derivatization products of (a) 500 pg/L IBHP and (b) 500 pg/L $^{2}[H_2]$-IBHP.

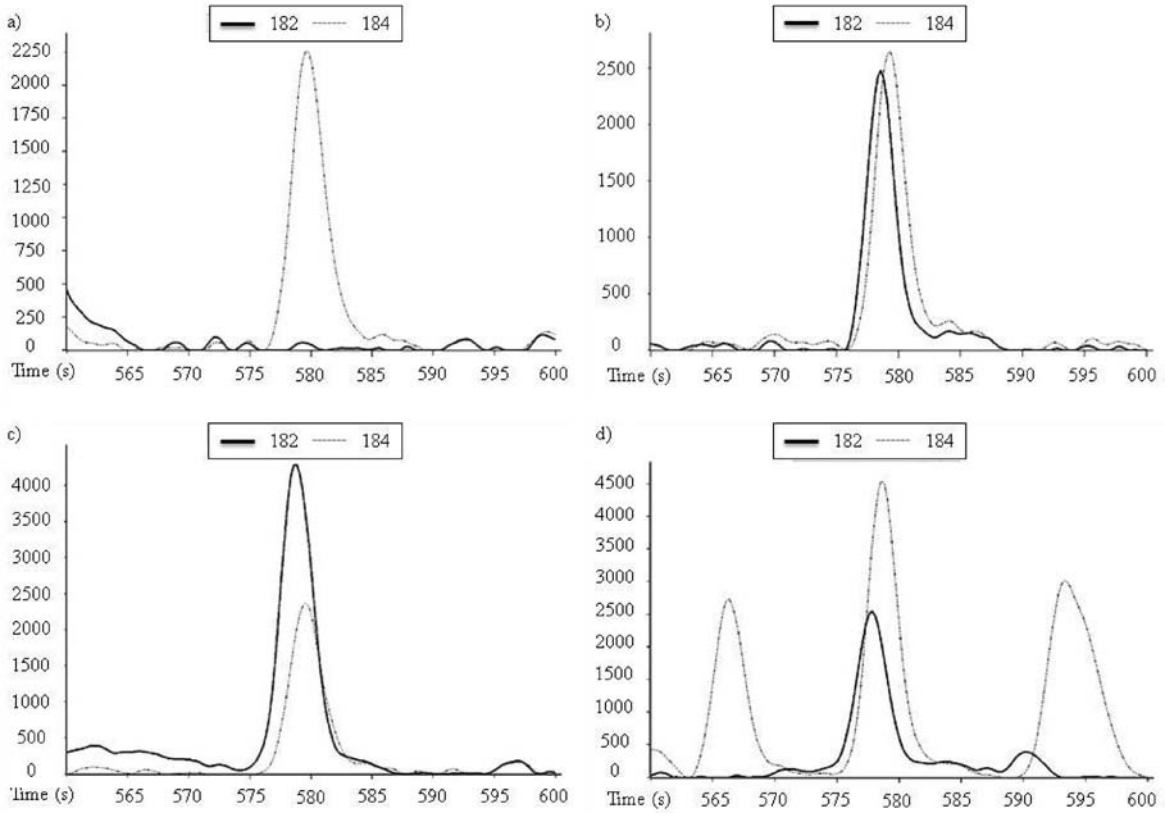

Figure 3. GC-TOF-MS chromatograms displaying *m/z* 182 and 184 ions of derivatized (a) model juice spiked with 0 ng/L IBHP and 500 ng/L $^{2}[H_2]$-IBHP, (b) 500 ng/L IBHP with 500 ng/L $^{2}[H_2]$-IBHP, (c) 1000 ng/L IBHP and 500 ng/L $^{2}[H_2]$-IBHP, and (d) Cabernet franc juice at veraison spiked with 500 ng/L $^{2}[H_2]$-IBHP.

lower dichloromethane concentrations, but this resulted in an increase in interfering peaks (data not shown).

Derivatization Method. Earlier studies of IBHP in grapes included GC-MS analyses on underivatized IBHP,[21,23] but these result in poor chromatographic behavior due to the presence of multiple H-bonding sites on IBHP.[23] We attempted to derivatize IBHP with multiple reagents, including trifluoroacetic acid (TFAA), HMDS, and Sweeley's reagent. TFAA and HMDS alone resulted in incomplete derivatization of IBHP (data not shown). Sweeley's reagent, composed of HMDS and TMCS in pyridine, has been previously recommended for derivatization of IBHP.[24] Following derivatization by Sweeley's reagent, direct injection of the reaction mixture showed a single derivative and no evidence of the IBHP starting material. Figure

2 shows the spectra of the qualifying ions for derivatized IBHP (top) and $^{2}[H_2]$-IBHP (bottom). The top spectrum is consistent with that of Hawksworth et al.[24] The silylated IBHP is unstable at room temperature, and no silylated peak could be detected after derivatized samples had been stored for 12−24 h at 25 °C. However, it was possible to rederivatize degraded samples without affecting accuracy (data not shown).

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 3. Panels a, b, and c of Figure 3 show chromatograms of model juice spiked with 0, 500, and 1000 ng/L IBHP, respectively, following extraction and derivatization. Figure 3d 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. Although the majority of samples were run on an FFAP column, we observed an interference for several late-season samples on the FFAP column. Therefore, samples from some late-season sampling 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, in which the heavier deuterated compound elutes after the undeuterated analogue. This was not the case for the 50% phenyl column, which exhibited the more common inverse isotope effect.

Figures of Merit: Recovery, Method Linearity, and Limits of Detection. The method linearity was evaluated using a five-point calibration curve (0, 100, 250, 500, and 1000 ng/L) prepared in model juice. Good linearity was observed (R^2 = 0.995, %RMSE = 5.7). Using Pallesen's method, the LOD for IBHP in model juice was calculated to be 35 ng/L. However, in real juice samples, the signal size for the deuterated standard was 2-fold higher than in model juice samples without a corresponding increase in noise. This effect is demonstrated in Figure 3. The loss of signal observed in model juices was likely a result of active binding sites on the glassware or SPE cartridge, a problem that is avoided with real juice samples. For two different real juice samples (n = 4) with native IBHP concentrations of 22 and 32 ng/L, we observed relative standard deviations of 9 and 16%. On the basis of these results, we estimate the limit of detection for IBHP in real juice samples using the optimized methodology to be <20 ng/L.

To validate that the responses for IBHP in real juice were comparable to that of model juice, a recovery experiment was performed on a Cabernet franc juice sample containing 93 ± 9 ng/L IBHP. The juice was spiked with the equivalent of 500 ng/L in triplicate, which should have resulted in a final concentration of 593 ng/L. We observed a final concentration of 619 ± 17 ng/L, or a recovery of 105%, indicating the model juice calibration curves were appropriate for use on real juice. Peak areas for the deuterated m/z 184 signal were not significantly different between the spiked and unspiked samples, suggesting that recovery at lower IBHP concentrations in real juice should be similar.

In summary, the use of mixed-mode cation-exchange SPE followed by silylation and one-dimensional GC-TOF-MS for quantification can achieve a 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 GC×GC-TOF-MS for quantification to achieve a similar LOD.[23]

Behavior of IBHP during the Growing Season. IBHP in berries was measured during the 2010 growing season in California Merlot and two New York Cabernet franc clones (CF1 and CF4). We attempted to sample at roughly 1−2 week intervals, with more frequent sampling around veraison for the Cabernet franc around harvest. However, some samples could not be collected at the expected interval because of unplanned pesticide spraying. Furthermore, some late-season samples had unidentified coelutions on both GC-TOF-MS and GC×GC-TOF-MS, which were recognizable by inappropriate qualifier ion ratios. For some samples it was possible to avoid these interferences by using a different column (RTX-50 vs FFAP), but for a few samples this was not possible, and these samples were not included.

A plot of IBHP concentration (pg/g) versus time for California Merlot is shown in Figure 4 and for the Cabernet

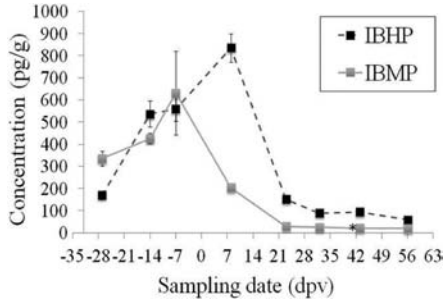

Figure 4. By-weight concentrations of IBMP and IBHP in California Merlot during ripening. The error bars represent the standard deviation of the biological duplicates. The X-axis represents date of sampling with respect to veraison (days postveraison, dpv). Data for CF1 and CF4 are shown in Supplementary Figure 1 of the Supporting Information. ∗ indicates no duplicate available.

franc samples in Supplementary Figure 1 of the Supporting Information. IBMP concentrations (pg/g) versus time are shown on the same plots. IBHP concentrations reach a maximum around veraison in all three sites. However, changes in w/w IBHP or IBMP concentration reflect both dilution of existing IBHP due to increasing berry size and formation or loss of IBHP in each berry.

To better appreciate the changes in IBHP occurring in the berry, time course profiles of IBHP per berry versus time are shown for all three sites in the top portion of Figure 5. Similar behavior for IBHP was observed for all three experiments: IBHP was detectable at the earliest sampling point (4 weeks preveraison, or approximately 3 weeks postbloom), increased to a maximum on a per-berry basis at 0−2 weeks postveraison, and then decreased during further ripening. For example, CF4 (Figure 5, right) had 12 pg IBHP/berry (21 pg/g) at 30 days preveraison, which increased to 178 pg/berry (254 pg/g) at veraison and decreased significantly by 43 days postveraison to 96 pg/berry (77 pg/g).

The highest IBHP concentration across all time points and experiments was observed in California Merlot (Figure 5, left) which peaked at 477 pg/berry (636 pg/g) at 8 days postveraison, or nearly double the maximum IBHP observed on both a per-berry and concentration basis in the New York Cabernet franc samples. IBHP decreased more rapidly in the Merlot samples, too, such that differences in final IBHP concentrations were lower. Due to the limited number of sites, it is not clear if the greater IBHP accumulation and faster degradation observed in California Merlot was due to the cultivar, the site, or some other factor.

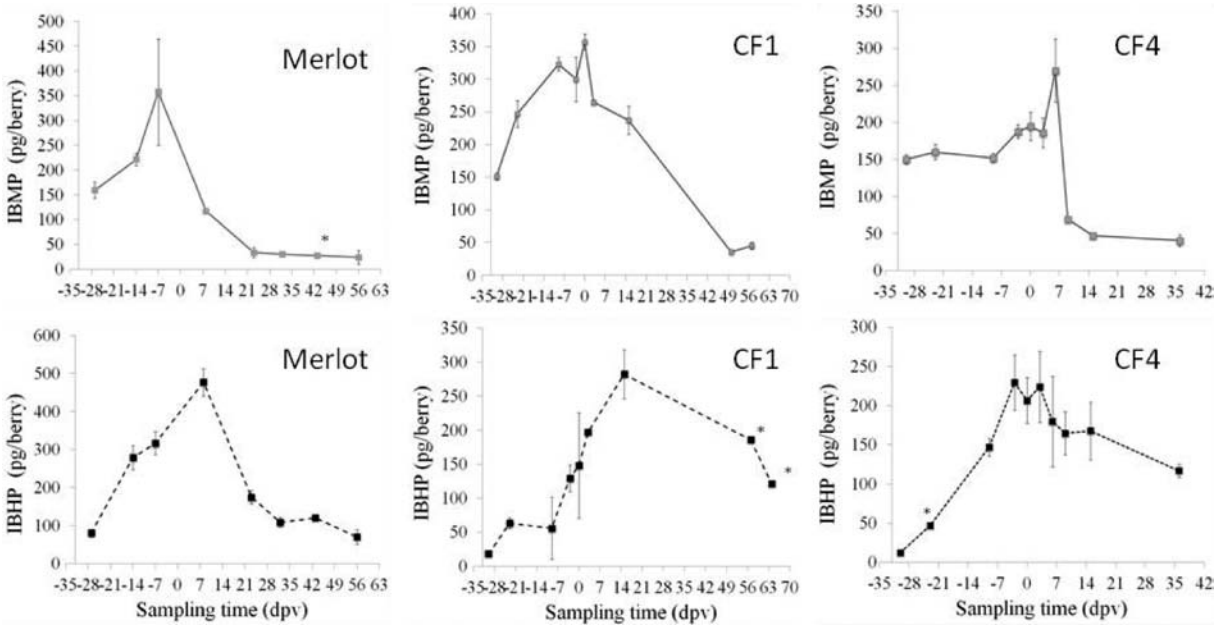

Figure 5. Per-berry concentrations of IBMP (top) and IBHP (bottom) during ripening over the 2010 growing season for CA Merlot (left), NY CF1 (middle), and NY CF4 (right). The *X*-axis represents date of sampling with respect to veraison (days postveraison, dpv). The error bars show the standard deviation of the biological duplicates. ∗ indicates no duplicate available.

This work represents the first detailed time course study on IBHP during the growing season, as previous studies have measured IBHP at only a single point before veraison and either one or two points after veraison.[21,23] In earlier work by our group, the IBHP concentration of New York State Cabernet franc at 11 days preveraison was 68 pg/mL of juice, comparable to the values observed 9 days preveraison in CF1 (80 pg/g), assuming the density of juice was ∼1 g/mL. However, concentrations of IBHP from the Merlot in our study were over an order of magnitude lower than the 15 nmol/kg (1824 pg/g) IBHP concentrations reported in Japanese Merlot at 40 days postbloom, presumably 2−3 weeks preveraison.[21]

Both of these previous studies on early-season IBHP sampled at 1−2 weeks preveraison were based on the expectation that the maximum IBHP concentration would be at the same time as the maximum IBMP concentration. An important observation from our current work is that it is evident that this assumption is incorrect and that the IBHP maximum occurs 0−2 weeks following veraison.

As a caveat, the samples analyzed in our current study, along with early studies, utilized grapes that were frozen and thoroughly macerated prior to analysis. The effects of tissue disruption on grape HP and MP concentrations are not well studied. Whereas the addition of deuterated standards during processing would account for sample losses due to, for example, enzymatic degradation, the methodology cannot detect if the measured pyrazines were present in the intact fruit or formed only following tissue disruption.

Behavior of IBMP during the Growing Season. Time course studies for IBMP per berry versus time at the three experimental sites are shown in Figure 5, top. Similar to previous studies,[6,15] IBMP increased early in the season, reached a maximum 1−2 weeks preveraison, and then decreased through harvest. The maximum concentration of IBMP observed across experimental sites was for the California Merlot and the Finger Lakes CF1, both of which peaked at 357 pg/berry. However, the Merlot had a higher concentration on a by-weight basis (628 pg/g) due to its smaller berry size. Not only is this concentration ca. 2-fold higher than the maximum IBMP concentrations observed for the other two sites, it is also higher than the maximum concentrations observed for Bordeaux cultivars in other time course studies.[6,15,18] This observation is somewhat surprising because higher IBMP concentrations are generally associated with cooler climates,[31] but the Central Valley of California is considerably warmer than both the Finger Lakes region of New York and other sites in which IBMP has been characterized, for example, Bordeaux. However, our observations are in agreement with the results from a recent multisite study in New York state on factors correlated with IBMP accumulation,[16] which observed that factors associated with growth, particularly high water availability and high temperatures, were best correlated with higher IBMP accumulation. The California Merlot used in our experiment was grown in irrigated conditions with high water availability (O. Kaye, Constellation Brands, personal communication).

At two of the sites (Merlot and CF1), the decrease in IBMP on a per berry basis appeared to begin the week prior to veraison, as is generally observed in the literature,[6,15,18] although the precise time of the onset of degradation was limited by the frequency of sampling. However, in CF4, no significant decrease in IBMP was observed until 10 days after veraison. It is unclear why this behavior occurred. Regardless, at the final CF4 sampling point, the IBMP concentrations had decreased to a range of concentrations, 32 pg/g or 40 pg/berry, previously observed in Bordeaux type grapes or varietal wines.[6]

Relationship of IBHP and IBMP during the Growing Season. IBHP is hypothesized to be both the precursor of IBMP preveraison[21] and a degradation product of IBMP postveraison.[23] We observed a preveraison increase in both IBHP and IBMP at all experimental sites, which is compatible with the hypothesis that IBHP is methylated by *VvOMT1* to form IBHP preveraison. The maximum IBHP per berry occurred ∼2 weeks after the IBMP maximum. Dunlevy et al.

had previously observed a decrease in *VvOMT1* expression between 8 and 10 weeks postflowering, correlating with a decrease in IBMP during the same time.[22] Thus, a potential explanation for the increase in IBHP at veraison is that a portion of the IBHP pool is no longer methylated to IBMP, allowing it to accumulate. Another explanation, not mutually exclusive, is that the increase in IBHP is due to demethylation of IBMP, a behavior previously observed in rats[24] and proposed to occur in grapes.[23]

Regardless of the biochemical mechanism, the earlier hypothesis that IBHP postveraison could be used as a proxy for maximum preveraison IBMP[23] is not valid because IBHP concentrations decrease during ripening rather than remain stable through ripening as previously hypothesized. A potential explanation is that a portion of the IBHP formed postveraison is glycosylated, as acid treatment of grape juice significantly increased IBHP concentrations by 33% in previous work.[23] To evaluate this hypothesis, we performed acid hydrolysis on the 57 day CF1 sample. We observed a nonsignificant increase ($p > 0.05$) in IBHP following the hydrolysis treatment, with 186 pg IBHP/berry observed initially and 210 pg IBHP/berry observed following hydrolysis.

Finally, we observed the highest concentrations of preveraison and maximum IBHP on a by-weight basis in the Merlot grapes, which was also the experimental site with the highest IBMP concentration (w/w). For example, at the earliest time point (27−30 days preveraison), the concentration of IBHP in Merlot was 81 pg/g, whereas the concentrations of IBHP in CF1 and CF4 were 18 and 12 pg/g, respectively. This suggests that differences in IBMP accumulation may be governed at least in part by differences in IBHP accumulation. However, differences in *VvOMT* activity could also explain variation in IBMP concentrations,[32] but this was not measured in the current study.

In conclusion, we have developed an SPE-GC-TOF-MS methodology for quantification of IBHP in grapes capable of 20 ng/L detection limits with a 100 mL sample size. IBHP concentrations peak approximately 2 weeks after maximum IBMP concentration and then decline during ripening. Because IBHP decreases after veraison, it does not appear possible to correlate IBHP at harvest with preveraison IBMP as previously hypothesized. The highest free IBHP concentrations were observed in grapes with the highest IBMP concentrations. Potentially, this may be because maximum IBMP accumulation is dependent on IBHP accumulation. Future studies that concurrently measure IBHP and IBMP concentration and *OMT* activity during the growing season across a range of sites or growing practices will be important for determining if variation in IBMP accumulation among sites or cultivars is governed by differences in IBHP accumulation as well as rates of IBHP methylation.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figure 1 and Supplementary Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: gls9@cornell.edu. Phone 1 (315) 787-2458. Fax: 1 (315) 787-2397.

Funding

This project was funded through the John S. Dyson and Canandaigua Wine Endowment Funds. S.H. was partially supported by a Cornell College of Agriculture and Life Sciences Extension-Outreach Assistantship.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the assistance of Justine Vanden Heuvel, Eric Shatt, and Céline Coquard-Lenerz (Cornell University) with sample collection from New York State and Oren Kaye (Constellation Brands) for providing samples from California.

■ REFERENCES

(1) Buttery, R. G.; Seifert, R. M.; Guadagni, D. G.; Ling, L. C. Characterization of some volatile constituents of bell peppers. *J. Agric. Food Chem.* **1969**, *17* (6), 1322−1327.

(2) Bayonove, C. R.; Cordonnier, R.; Dubois, P. 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*, 75−78.

(3) Murray, K. E.; Whitfield, F. B. The occurrence of 3-alkyl-2-methoxypyrazines in raw vegetables. *J. Sci. Food Agric.* **1975**, *26*, 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. Agric. Food Chem.* **1970**, *18* (2), 246−249.

(5) Allen, M. S.; Lacey, M. J.; Harris, R. L. N.; Brown, W. V. Contribution of methoxypyrazines to Sauvignon blanc wine aroma. *Am. J. Enol. Vitic.* **1991**, *42* (2), 109−112.

(6) Roujou de Boubee, D.; Van Leeuwen, C.; Dubourdieu, D. Organoleptic impact of 2-methoxy-3-isobutylpyrazine on red Bordeaux and Loire wines. Effect of environmental conditions on concentrations in grapes during ripening. *J. Agric. Food Chem.* **2000**, *48* (10), 4830−4834.

(7) Preston, L. D.; Block, D. E.; Heymann, H.; Soleas, G.; Noble, A. C.; Ebeler, S. Defining vegetal aromas in Cabernet Sauvignon using sensory and chemical evaluations. *Am. J. Enol. Vitic.* **2008**, *59*, 137−145.

(8) Ebeler, S. E.; Thorngate, J. H. Wine chemistry and flavor: looking into the crystal glass. *J. Agric. Food Chem.* **2009**, *57*, 8098−8108.

(9) Marais, J. Sauvignon blanc cultivar aroma − a review. *S. Afr. J. Enol. Vitic.* **1994**, *15* (2), 41−45.

(10) Bogart, K.; Bisson, L. Persistence of vegetal characters in winegrapes and wine. *Practical Vineyard Winery* **2006**, *86*, 13−20.

(11) Allen, M. S.; Lacey, M. J.; Boyd, S. Determination of methoxypyrazines in red wines by stable isotope dilution gas chromatography-mass spectrometry. *J. Agric. Food Chem.* **1994**, *42* (8), 1734−1738.

(12) Sala, C.; Busto, O.; Guasch, J.; Zamora, F. 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. Agric. Food Chem.* **2004**, *52*, 3492−3497.

(13) Sala, C.; Guasch, J.; Zamora, F. *Factors Affecting the Presence of 3-Alkyl-2-methoxypyrazines in Grapes and Wines. A Review*; Universitat Rovira i Virgili: Tarragona, Spain, 2004.

(14) Scheiner, J. J.; Sacks, G. L.; Vanden Heuvel, J. E. How viticultural factors affect methoxypyrazines. *Wines Vines* **2009**, November.

(15) Ryona, I.; Pan, B. S.; Intrigliolo, D. S.; Lakso, A. N.; Sacks, G. L. Effects of cluster light exposure on 3-isobutyl-2-methoxypyrazine accumulation and degradation patterns in red wine grapes (*Vitis vinifera* L. cv. Cabernet franc). *J. Agric. Food Chem.* **2008**, *56* (22), 10838−10846.

(16) Scheiner, J. J.; Vanden Heuvel, J. E.; Pan, B.; Sacks, G. L. Modeling impacts of viticultural and environmental factors on 3-

isobutyl-2-methoxypyrazine in Cabernet franc grapes. *Am. J. Enol. Vitic.* **2012**, *63* (1), 94−105.
(17) Marais, J.; Hunter, J. J.; Haasbroek, P. D. Effect of canopy microclimate, season and region on Sauvignon blanc grape composition and wine quality. *S. Afr. J. Enol. Vitic.* **1999**, *20*, 19−30.
(18) Koch, A.; Ebeler, S. E.; Williams, L. E.; Matthews, M. A. Fruit ripening in *Vitis vinifera*: light intensity before and not during ripening determines the concentration of 2-methoxy-3-isobutylpyrazine in Cabernet Sauvignon berries. *Physiol. Plant.* **2012**, *145* (2), 275−285.
(19) Rizzi, G. P. Formation of methoxypyrazines in reactions of 2(1*H*)-pyrazinones with naturally occuring methylating agents. *J. Agric. Food Chem.* **1990**, *38*, 1941−1944.
(20) Hashizume, K.; Tozawa, K.; Hiraga, Y.; Aramaki, I. Purification and characterization of a *O*-methyltransferase capable of methylating 2-hydrozy-3-alkylpyrazine from *Vitis vinifera* L. (cv. Cabernet Sauvignon). *Biosci., Biotechnol., Biochem.* **2001**, *65* (10), 2213−2219.
(21) Hashizume, K.; Tozawa, K.; Endo, M.; Aramaki, I. *S*-Adenosyl-L-methionine-dependent O-methylation of 2-hydroxy-3-alkylpyrazine in wine grapes: a putative final step of methoxypyrazine biosynthesis. *Biosci., Biotechnol., Biochem.* **2001**, *65* (4), 795−801.
(22) Dunlevy, J. D.; Soole, K. L.; Perkins, M. V.; Dennis, E. G.; Keyzers, R. A.; Kalua, C. M.; Boss, P. K. Two *O*-methyltransferases involved in the biosynthesis of methoxypyrazines: grape-derived aroma compounds important to wine flavor. *Plant Mol. Biol.* **2010**, *74*, 77−89.
(23) Ryona, I.; Leclerc, S.; Sacks, G. L. Correlation of 3-isobutyl-2-methoxypyrazine to 3-isobutyl-2-hydroxypyrazine during maturation of bell pepper (*Capsicum annuum*) and wine grapes (*Vitis vinifera*). *J. Agric. Food Chem.* **2010**, *58* (17), 9723−9730.
(24) Hawksworth, G.; Scheline, R. R. Metabolism in rat of some pyrazine derivatives having flavor importance in foods. *Xenobiotica* **1975**, *5* (7), 389−399.
(25) 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*, 4088−4090.
(26) Gerritsma, D. A.; Brindle, I. D.; Jones, T. R. B.; Capretta, A. Preparation of labelled 2-methoxy-3-alkylpyrazines: synthesis and characterization of deuterated 2-methoxy-3-isopropylyrazine and 2-methoxy-3-isobutylpyrazine. *J. Label. Compd. Radiopharm.* **2003**, *46* (3), 243−253.
(27) Sweeley, C. C.; Bentley, R.; Makita, M.; Wells, W. W. Gas-liquid chromatography of trimethyl derivatives of sugars and related substances. *J. Am. Chem. Soc.* **1963**, *85*, 2497−2507.
(28) Mac Berthoeux, P.; Brown, L. C. *Statistics for Environmental Engineers*, 2nd ed.; CRC: Boca Raton, FL, 2002.
(29) Ryona, I.; Pan, B. S.; Sacks, G. L. Rapid measurement of 3-alkyl-2-methoxypyrazine content of winegrapes to predict levels in resultant wines. *J. Agric. Food Chem.* **2009**, *57*, 8250−8257.
(30) López, R.; Gracia-Moreno, E.; Cacho, J.; Ferrreira, V. 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. Chromatogr., A* **2011**, *1218* (6), 842−848.
(31) Allen, M. S.; Lacey, M. J. Methoxypyrazine grape flavour: influence of climate, cultivar, and viticulture. *Vitic. Enol. Sci* **1993**, *48*, 211−213.
(32) Vallarino, J. G.; Lopez-Cortes, X. A.; Dunlevy, J. D.; Boss, P. K.; Gonzalez-Nilo, F. D.; Moreno, Y. M. Biosynthesis of methoxypyrazines: elucidating the structural/functional relationship of two *Vitis vinifera* *O*-methyltransferases capable of catalyzing the putative final step of the biosynthesis of 3-alkyl-2-methoxypyrazine. *J. Agric. Food Chem.* **2011**, *59* (13), 7310−7316.