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ARTICLE

Rapid Measurement of 3-Alkyl-2-methoxypyrazine Content of Winegrapes To Predict Levels in Resultant Wines

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We describe an optimized protocol for analysis of the herbaceous smelling 3-alkyl-2-methoxypyrazines (MPs) in whole berries that predicts MPs in resultant red wines. Berries are homogenized by bead-milling with a deuterated standard prior to headspace solid phase microextraction (HS-SPME) and quantification by two-dimensional gas chromatography time-of-flight-mass-spectrometry (GC×GC−TOF-MS). In the case of 3-isopropyl-2-methoxypyrazine (IPMP), GC×GC−TOF-MS successfully resolved interferences that coeluted with the analyte in the first dimension. HS-SPME parameters (pH, queue time, incubation time, extraction time, extraction temperature) were optimized by a statistical experimental design. Good method accuracy was observed (consistent ratio of unlabeled analyte to labeled standard) at 10 min extraction times when 80 °C extraction temperatures were employed, although increasing sensitivity was observed for longer extraction times (up to 140 min). Standard addition of 3-isobutyl-2-methoxypyrazine (IBMP) and IPMP into preveraison and harvest ripe berry matrices showed good linearity (r^2 >0.99 in all cases), with limits of detection ranging from 0.6 to 1.8 pg/g. The protocol was validated by comparing IBMP in 16 lots of Cabernet Franc berries (range = undetectable to 18.4 pg/g) to the resulting wines (range = undetectable to 14.5 pg/g). Berry and wine MP content were strongly correlated, (r^2 = 0.97, p < 0.0001). Following correction for CO_2 loss, the observed concentration of IBMP in wines was 67 ± 13% of the IBMP concentration observed in berries.

KEYWORDS: Alkylmethoxypyrazines; IBMP; IPMP; Cabernet Franc; SPME; GC×GC−TOF-MS; berry homogenate

INTRODUCTION

The 3-alkyl-2-methoxypyrazines (MPs) are potent odorants possessing herbaceous, musty and unripe aromas (*1*). Although MPs are found in many plant species, the majority of reports on MPs in recent years have focused on its presence in wine grapes (*Vitis vinifera*) and role in wine aroma. Several authors have reported a correlation of "vegetal" or "bell pepper" character and MP concentrations in finished wines (*2*−*4*), although this is not a universal observation (*5*). The best studied MP in wine and winegrapes is 3-isobutyl-2-methoxypyrazine (IBMP), and to a lesser extent 3-isopropyl-2-methoxypyrazine (IPMP) (*2*). The sensory thresholds of IBMP and IPMP in red wines are reported to be 10−16 pg/g (*1*, *2*, *6*) and 1−2 pg/g (*7*), respectively, and the appearance of these MPs in table wines in excess of threshold is not an infrequent occurrence (*8*).

Since strong herbaceous character is generally considered undesirable in red wines, studies on the impact of viticultural factors on MPs in grape berries are of current interest (*9*). Recent studies have considered the impact of cluster light exposure (*10*, *11*), water status (*12*), planting density (*12*), or vine growth (*13*) on MP levels in harvested grapes. Measurement of MPs in grapes has also been used to monitor changes of MPs within or between growing seasons (*2*, *11*, *14*−*16*).

Considering the low sensory thresholds of MPs in wines, an optimized analysis for MPs in grapes should be able to achieve low pg/g detection thresholds. Early reports (*17*−*20*) used a distillation pretreatment, followed by extraction onto a strong-cation exchange (SCX) resin, elution with alkaline buffer, liquid−liquid extraction (LLE) and finally quantification by GC−MS. More recent MP analyses have replaced the tedious distillation and SCX steps with a single C-18 SPE (*21*, *22*) or nonpolar LLE (*6*, *23*) cleanup and preconcentration step. Alternatively, the use of headspace solid-phase microextraction (HS-SPME) for solventless extraction and preconcentration of MPs has become increasingly popular. Compared to LLE and SPE approaches, HS-SPME sample preparation is rapid, is readily automated, and demands smaller sample sizes. Several groups have investigated the impact of sample parameters on HS-SPME extraction of MPs from wine samples (*24*−*30*). These reports have considered, among other parameters, the impact of ethanol concentration (*26*−*28*), extraction time and temperature (*24*−*30*), and sample pH (*25*−*27*, *30*).

While the majority of reports have focused on measuring MPs in wines, it is frequently advantageous or necessary to measure MPs in grapes. Measuring MPs in grapes avoids the expense,

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Table 1. Description and Range of Optimized Extraction Parameters

parameters	values used	descriptions
pH	2, 5.5, 9	sample's pH adjusted with NaOH solution
TEMP	30, 55, 80 °C	the setting for the incubation and extraction temperature on the heating block
QUE	0, 12, 24 h	sample-queue time on the tray at ambient temperature prior to incubation
INC	10, 20, 30 min	sample-incubation time in the heating block before exposure of the SPME fiber
EXT	10, 35, 70, 140 min	sample-extraction time in the heating block after exposure of the SPME fiber

time period	QUE	INC[a]	EXT[a]
exposed to SPME fiber?	no	no	yes
temp	ambient (~25 °C)	TEMP	TEMP

[a] Agitation speed was set at 650 rpm.

time, sample sizes and potential variability associated with vinification. Ideally, the concentration of MPs measured in fruit should predict the eventual MP concentration in finished wine. However, careful inspection of previous reports reveals a poor correlation between MPs measured in juice or must samples and MPs in wine. Kotseridis et al. (*23*) reported inconsistencies between MP measured by LLE in grapes and their resulting wines depending on site, cultivar, and the disease condition of the grapes. A similarly poor correlation has been observed with HS-SPME extraction (*10*, *12*) although the relationship was not formally characterized. These discrepancies are likely because MPs in berries are located primarily in the skins (*31*), and the MPs present in juice immediately following crushing thus represents only a fraction of the MPs extractable from berries.

Also, in contrast to the rich literature on optimization of HS-SPME for analysis of MPs in wines, there is only a single report regarding optimization of HS-SPME parameters for berries, juice, or must (*32*). In this report, Sala et al. reported that optimal extraction of MPs from juices was achieved with a mixed-mode fiber (PDMS-DVB), a 4 h extraction time at 30 °C, and addition of at least 0.3 g/mL of NaCl. However, statistical experimental design was not employed, i.e. each parameter was optimized while keeping other parameters constant.

Using a statistical experiment design, we have developed an optimized SPME methodology for rapid quantification of MPs in whole berries with small sample size demands. The optimized method was validated by comparing MP concentrations in Cabernet Franc berries collected from 16 vineyard sites to the MP contents of the resulting wines.

MATERIALS AND METHODS

Analytical Reagents. NaCl, NaOH, $CaCl_2$ and EDTA were purchased from Fisher Scientific (Atlanta, GA). IBMP (99%) and IPMP (97%) were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified by Milli-Q system from Millipore (Bedford, MA). [$^2H_2$]-IBMP was synthesized in our laboratory according to the method of Kotseridis et al. (*33*).

Generalized Protocol for Whole Berry Sample Preparation. Frozen whole berries were placed in 20 mL PET vials along with 1.0 cm stainless-steel balls. Vials were loaded onto a mechanical bead miller (2000 Geno/Grinder, SPEX Certiprep, Metuchen, NJ). The berries were pulverized at a rate of 1650 strokes/min for 2 min. While the HS-SPME extraction required only 5 g of berry homogenate, in practice, 25−50 g of berries was processed simultaneously to mitigate berry-to-berry variation. The homogenate from multiple vials was merged, weighed, and diluted 50% w/w with 0.1 M EDTA (adjusted to pH = 7.5 with NaOH) to facilitate sample handling. To prevent potential enzymatic reactions, 5% w/w $CaCl_2$ was then added to the diluted homogenate. The diluted homogenate (10 g) was weighed into a 20 mL amber SPME vial (Sigma-Aldrich, St Louis, MO) along with 3 g of NaCl and 20 μL of internal standard of 2.5 ppb [$^2H_2$]-IBMP aqueous solution. While transferring homogenate to the SPME vial, the insoluble solids of the mixture did not stay suspended without constant agitation. Therefore, the mixture was stirred by a magnetic stir-bar throughout the transfer process.

Statistical Experiment Design for Method Optimization. A center composite face-centered (CCF) model was used to optimize HS-SPME parameters. A statistical package (MODDE Version 6.0, Umetrics Inc.) was used to generate the model and evaluate the resulting response data. Five factors were examined in the CCF model: sample pH (PH), extraction temperature (TEMP), queue time (QUE), incubation time (INC), and extraction time (EXT). The description and parameter range of each factor are listed in **Table 1**, and the full run list is presented in the Supporting Information (Table S1). During INC and EXT periods, the sample was agitated on the autosampler heating block at 650 rpm at temperature, TEMP. During the QUE period, the sample was held at ambient temperature. The initial experiment only used 10, 35, and 70 min extraction times, for a total of 29 runs including 3 center-point replicates. To demonstrate complete extraction of IBMP at 80 °C at the region beyond EXT = 70 min, 8 additional runs were performed with EXT = 140 min, for a total of 37 runs. The sample order was established by the CCF model to avoid bias caused by instrumental drift.

Cabernet Franc berries were sourced from a local vineyard (Geneva, NY). Berries were harvested at the IBMP maximum, 47 days post-bloom (*11*). Two hundred fifty grams of frozen whole berries were homogenized by bead milling as described above. For each optimization run, 5 g of berry homogenate was accurately weighed into a 20 mL SPME vial and kept frozen at −80 °C prior to analysis. On the day of analysis, frozen samples were thawed at room temperature for 1 h. Subsequently, 5 g of the EDTA solution, 0.5 g of $CaCl_2$, and the [$^2H_2$]-IBMP internal standard were added to the SPME vial. The vial was then capped and vortexed for 10 s. The pH of the samples was adjusted with 20% NaOH solution. Any discrepancy in volume created by this adjustment was corrected with H_2O.

HS-SPME Extraction and GC×GC−TOF-MS. HS-SPME analyses were performed by a LEAP CombiPAL Autosampler (Carrboro, NC). A 2 cm, 50/30 μm divinylbenzene−carboxen−polydimethylsiloxane (DVB/CARB/PDMS) SPME fiber was used for all experiments (Supleco, Bellafonte, PA). Quantification was performed by two-dimensional comprehensive gas chromatography, coupled to time-of-flight mass spectrometry (GC×GC−TOF-MS) (LECO Pegasus 4D Leco Corp, St. Joseph, MI). SPME injections were splitless with a desorption temperature of 270 °C. The first capillary column (30m × 0.25 mm × 0.50 μm) was a RTX5 (Restek, Bellefonte, PA), and the second column (2.5m × 0.10 mm × 0.10 μm) was a VF-WAXms (Varian, Palo Alto, CA). Helium was used as a carrier gas at a flow rate of 1 mL/min. The temperature program was as follows: Initial hold for 5 min at 40 °C, followed by 10 °C/min ramp to 110 °C; then, 2 °C/min to 147 °C, no hold; then 40 °C/min to 260 ^{0} C, 15 min hold. The starting temperature for GC2 was 70 °C, hold for 8 min, then identical to GC1 for the remainder of the run. The GC×GC modulation time was 3 s, resulting in approximately 3−4 samples across each first dimension peak. The MS transfer line temperature was 230 °C. The TOF-MS was operated in EI mode. The ionization energy was 70 eV. The electron multiplier was set to 1680 V. The TOF-MS data was stored at an effective acquisition rate of 120 Hz. Data processing was carried out by ChromaTOF software. The qualifier ions were m/z = 124, 151, 166 for IBMP and m/z 126, 153, 168 for [$^2H_2$]-IBMP. The quantifier ions were m/z = 124 and 126, respectively. For IPMP, the qualifier ions were m/z = 137, 124, and 152 and quantifier ion was m/z = 137. Prior to these analyses, calibration curves were generated from IBMP and IPMP standards (n = 6) prepared over a range of 0−200 pg/g in EDTA/NaOH (pH 7.5). Weighted ($1/X$) linear regressions of [124]/[126] ions vs IBMP concentration and

[137]/[126] ions vs IPMP concentration were used for quantification of berry samples. Calibration curve slopes for IBMP and IPMP in aqueous buffer were similar to those observed in berry matrices.

Limit of Detection for IBMP and IPMP in Berry Matrices. Limits of detection (LOD) were determined by a 6-point standard addition of IBMP and IPMP to both preveraison and harvest ripe Pixie berries (*V. vinifera* cv. Pixie). The standard addition range was 0.5−200 pg/g for preveraison berries and 0−200 pg/g for harvest-ripe berries. Pixie berries were chosen for their availability and low levels of endogenous MPs. Samples were prepared by bead milling in 3 replicates per addition level, as described above. Twenty microliters of 2.5 ppb [$^2H_2$]-IBMP was used as the internal standard. LOD were calculated by the method of Pallesen (*34*). Briefly, the error associated with analytical measurements is assumed to be due to both constant background noise, σ_i, with variance σ_i^2 and variable noise, Conc × σ_d, whose variance, Conc^2 × σ_d^2, scales proportionally with the square of concentration, Conc. The constant background σ_i defines the inherent background noise for a blank measurement. The total variance (σ_T^2) at a given concentration is therefore the sum of both the constant and signal-dependent variances, or

$$\sigma_T^2 = \sigma_i^2 + \text{Conc}^2 \times \sigma_d^2 \quad (1)$$

The total variance, σ_T^2, can be determined at different concentrations by replicate measurements. A linear regression of σ_T^2 vs Conc^2 then reveals σ_i^2 (y-intercept) and σ_d^2 (slope). The limit of detection is calculated as 3× the signal-independent noise, σ_i. We employed a $1/X$ weighting factor for the linear regressions. The advantages of the Pallesen approach are that it readily incorporates multiple measurements at different concentrations, and that it decouples the true background noise from noise that increases with signal size.

Correlation of MP Measurements in Grapes and Wines: Grape Samples, Juice Samples, and Winemaking. Cabernet Franc (*Vitis vinifera* sp.) grapes were hand-harvested from 16 vineyard sites located in Long Island and Finger Lakes AVAs (New York State). Fruit was harvested from multiple vines at each site, with average yield per vine varying from 1.2 to 10.6 kg. These 16 sites encompassed a range of viticultural practices, clonal selections, and growing conditions. Basic juice parameters (pH, Brix, and TA) were measured. Prior to fermentation, whole berries (∼1 kg) were randomly collected from each site and kept frozen at −80 °C for later whole berry analysis. The remaining grapes were destemmed, crushed, and sulfited (50 ppm as SO_2) into a glass container fitted with an airlock. Immediately following crushing, 10 mL of juice was sampled into a SPME vial for later analysis. Similar basic red winemaking procedure was applied to all 16 juice samples producing a range of 1 gallon to 5 gallon finished wines. Crushed grapes or musts were fermented to dryness (<1 g/L residual sugar) with Lalvin ICV GRE yeast from Lallemand (Santa Rosa, CA). At the end of alcoholic fermentation (7−14 days), wines were pressed off into carboys for malolactic fermentation (Enoferm Alpha, Lallemand (Santa Rosa, CA). Sixty parts per million of SO_2 was added to the finished wines, and the wines were cold-stabilized at 2 °C for 60 days prior to wine analysis.

Whole berries from each site were analyzed by the previously optimized protocol. Free-run juice samples were analyzed by a similar protocol to whole berries, except that no dilution buffer was employed. For wine analysis, 5 mL of wine was diluted with 5 mL of Milli-Q water. For wine analyses, HS-SPME conditions similar to those optimized by other groups were used (*26*, *29*, *30*). Three grams of NaCl and 20 μL of internal standard of 2.5 ppb [$^2H_2$]-IBMP were added prior to analysis. The extraction temperature was 40 °C, instead of the 80 °C applied in whole berry and juice analyses. Similar to whole berry homogenates, LOD were determined for wines by standard addition, and calculated by the method of Pallesen.

RESULTS AND DISCUSSION

Rapid Whole Berry Sample Preparation. As opposed to previous accounts of HS-SPME extraction of MPs from juice or must (*4*, *26*, *32*), our current protocol involves homogenization of whole berries using a small volume bead miller (Geno/Grinder 2000). Bead mills are commonly used for homogenization of small tissue samples, e.g. in preparation for DNA or RNA extraction. In comparison to hand crushing and pressing small samples of berries through cheesecloth or use of a blender, we observed that bead milling is more rapid (<2 min) and reproducible, and minimizes sample losses. While we only consider the use of the bead mill in the analysis of IBMP and IPMP in this report, we expect that many other compound classes should be accessible. There are caveats for the use of this bead milling to produce whole berry homogenates. First, the small 20 mL PET vials may be inefficient in situations where large samples are needed to minimize a large degree of berry-to-berry variation. In these circumstances, it would be more efficient either to use a larger volume vial (50 mL) or to combine samples from multiple vials. The latter approach was used in our sample preparation. Second, the use of stainless steel beads results in homogenization of seeds in addition to mesocarp and exocarp. The extractability of MPs from seeds under normal red winemaking conditions is not established, so it is currently unclear if seed maceration assists or detracts from the reproducibility of IBMP measurements. However, since IBMP in harvest-ripe Cabernet Sauvignon has been reported to exist predominantly in the skins (95%), with a small fraction in the seeds (4%) (*31*), it is unlikely that any major discrepancy resulted from maceration of seeds. Although not investigated in this study, Teflon beads could be substituted for stainless steel beads if it is desirable to keep the seeds intact.

Separation Power of HS-SPME−GC×GC−TOF-MS. The base peak of the EI-MS mass spectrum of IPMP is m/z = 137, and is the most frequently used quantifier ion in previous reports on IPMP quantification in wines or musts (*4*, *26*, *35*). The IPMP elution region of a representative HS-SPME−GC×GC−TOF-MS contour plot for m/z = 137 is shown in **Figure 1**, left. The sample, preveraison Pixie grape homogenate, contains only trace levels (0.7 pg/g) of native IPMP as calculated in later experiments by standard addition. The IPMP peak coeluted in the first dimension 5% phenyl column with two interfering compounds. The first interference, present in blanks and labeled B in **Figure 1**, was tentatively identified as a silylated phenyl compound (m/z = 267, 126, 193) and was likely derived from either column or septum bleed. The second interference, labeled MT in **Figure 1**, was tentatively identified as a monoterpene (m/z = 136, 121, 93) and was grape derived. These interferences are resolved by the second dimension WAX column. Inspection of the unfolded GC×GC chromatogram (**Figure 1**, right) reveals that coelutions in the first GC dimension occur for both the m/z = 137 and the widely used qualifier ion, m/z = 152. Coelutions in the m/z = 137 and 152 traces were also observed when a WAX column was used in the first GC dimension (data not shown).

The unfolded HS-SPME−GC×GC−TOF-MS elution region for the [$^2H_2$]-IBMP standard (16.6 pg/g) and native IBMP (4.7 pg/g, determined by standard addition) from the same grape sample is shown in **Figure 2**. The m/z = 126 trace is derived solely from the [$^2H_2$]-IBMP standard, while the m/z = 124 trace has contributions from both the deuterated and native IBMP. The [$^2H_2$]-IBMP elutes slightly ahead of the native IBMP in the first dimension GC. However, during peak integration of calibration standards or real samples, the m/z = 124 and m/z = 126 signals are integrated over the full window covering the elution times of both native and deuterated species. Because the concentration of [$^2H_2$]-IBMP added to each sample is constant, the contribution of the [$^2H_2$]-IBMP to the m/z = 124 trace is also constant. Compared to IPMP, no significant coelutions are observed in the first dimension GC for IBMP. A small interference is observed centered at GC-1 retention time = 1109 s, and was tentatively identified as a siloxane derived from column bleed.

Statistical Experiment Design for Method Optimization. Most studies on MP analysis in wines have reported superior results with a three-phase fiber (PDMS/DVB/CARB) (*26*, *27*), while an

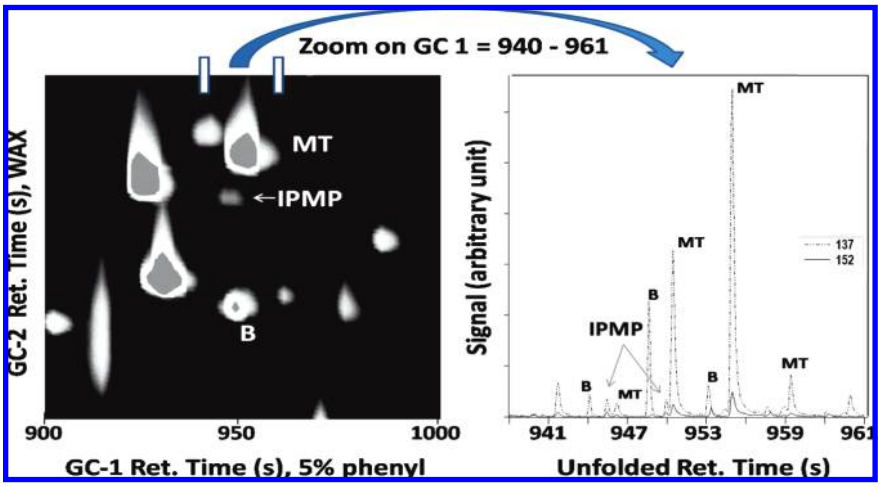

Figure 1. (Left) Contour plot (m/z = 137) of region of IPMP elution from HS-SPME−GC×GC−TOF-MS analysis of preveraison Pixie berries. IPMP concentration was 0.7 pg/g, calculated by standard addition. Two prominent interferences are visible in the first GC dimension, tentatively identified as a monoterpene (MT) and column bleed (B), but are resolved by GC×GC. (Right) Unfolded chromatogram, zoomed in on 940−961 s region. The m/z = 137 quantifier ion (- - -) and m/z = 152 qualifier ion (—) are shown.

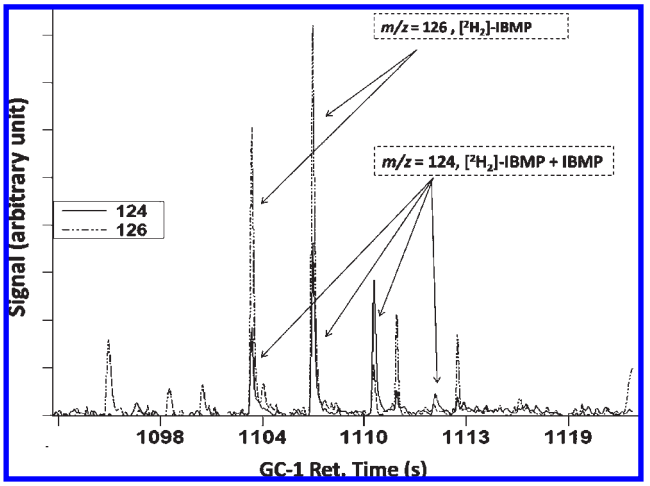

Figure 2. Unfolded chromatogram from SPME−GC×GC−TOF-MS analysis of preveraison Pixie berries, showing region of IBMP elution. The m/z = 126 trace (- - -) is derived from the 16.6 pg/g [$^2H_2$]-IBMP labeled standard. The m/z = 124 trace (—) has contributions from both the native IBMP (4.7 pg/g) and [$^2H_2$]-IBMP. The contribution of [$^2H_2$]-IBMP to the native IBMP signal is constant and accounted for in the calibration curves, as described in the text.

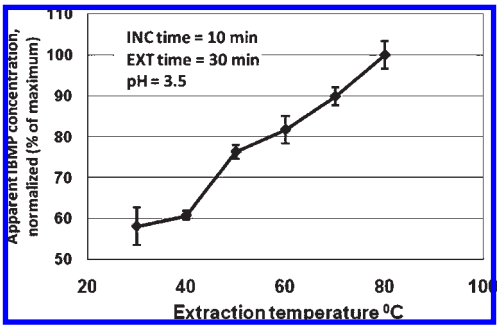

Figure 3. Apparent IBMP concentration in Cabernet Franc berries quantified in analytical duplicate at different extraction temperatures. IBMP concentrations are normalized to 80 °C = 100%.

early report on MP analysis in juice used a single phase PDMS fiber (*32*). Our preliminary investigations of commercially available fibers showed that the mixed phase PDMS/DVB/CARB fiber gave consistently higher responses than single phase PDMS and polyacrylate (PA) fibers (data not shown). Single phase DVB and CARB fibers were not commercially available for comparison. Thus, the mixed phase fiber was selected for the optimization study. Previous reports on adjusting ionic strength prior to SPME in grapes and other homogenates have also consistently indicated that addition of NaCl to saturation will improve SPME recovery via salting-out (*26*, *32*). Therefore, 3 g of NaCl was added to all samples.

A preliminary evaluation of the impact of HS-SPME extraction temperature (TEMP) on [124]/[126] ratio revealed that the measured IBMP concentration increases linearly with increasing TEMP (**Figure 3**). The IBMP concentration was determined from the ratio of the IBMP analyte, m/z = 124, to the [$^2H_2$]-IBMP standard, m/z = 126, observed at each temperature. Because IBMP is located primarily in the skins (*31*) IBMP must first migrate into the extracellular matrix before it can volatilize into the headspace and adsorb onto the SPME fiber. In contrast, [$^2H_2$]-IBMP is spiked into the sample, and therefore exists in the extracellular matrix from the beginning of the extraction. Thus, the increasing [124]/[126] ratio reflects the equilibration of the endogenous IBMP with the [$^2H_2$]-IBMP standard in the extracellular matrix and the accuracy of the measurement.

To determine the impact of extraction parameters on method sensitivity, the outputs of the 37 runs were fit by partial least squares (PLS) modeling, with the [$^2H_2$]-IBMP peak areas (m/z = 126) as the dependent variable. The deuterated sample signal was utilized because it did not need to diffuse from the skin cells to be accessible to the headspace. Values of PLS-VIP (variable importance in the projection) and PLS-regression coefficients were determined for 20 factors. Those factors with a VIP value less than 1 and small or insignificant regression coefficient were excluded from future models (*36*). Four factors were determined to be significant to the response: TEMP, EXT, TEMP*EXT, and EXT*EXT, and are listed in **Table 2**. Refitting the data with these 4 factors improved the average Q^2 (cross-validated R^2) from 0.619 to 0.776. The average R^2 value decreased slightly (0.894 to 0.857) with the refined model. In summary, the responses are well modeled by only the SPME extraction temperature (TEMP) and SPME extraction time (EXT) and their interaction terms,

Table 2. PLS-VIP and PLS-Regression Coefficients of Factors with Significant Impact on [$^2H_2$]-IBMP (m/z = 126) Signal

no.	factors	PLS-VIP	PLS regression coeff	p values
1	TEMP	2.457	0.5482	$p < 0.0001$
2	EXT	2.303	0.4647	$p = 0.0003$
3	EXT*EXT	1.424	0.1454	$p = 0.1619$
4	TEMP*EXT	1.266	0.2430	$p = 0.0065$

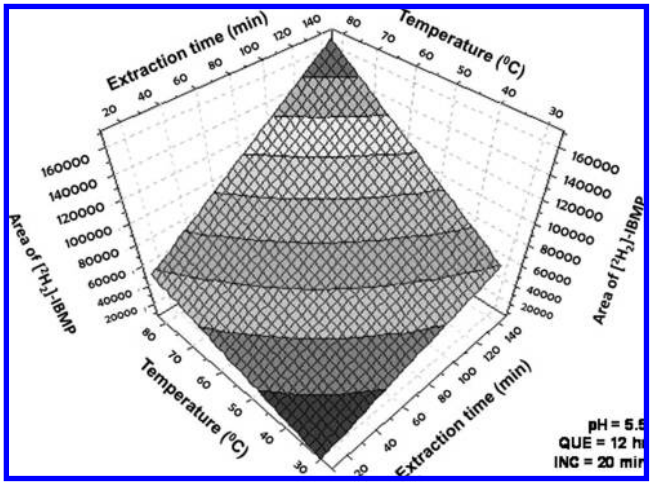

Figure 4. Response surface plot of TEMP versus EXT with the internal standard signal, ([$^2H_2$]-IBMP (m/z = 126) as the response.

with PH, QUE, and INC and other second order terms having no effect on recovery of the deuterated standard.

A 3-dimensional surface plot of TEMP versus EXT with [$^2H_2$]-IBMP signal as the response is shown in **Figure 4**, with PH, QUE, and INC values at center values. As expected from our early analyses (**Figure 3**), maximum SPME recovery of [$^2H_2$]-IBMP from berry homogenate was achieved at TEMP = 80 °C for all EXT times, considerably higher than the 30 °C suggested as optimal for HS-SPME analyses of juice by Sala et al. (*32*). Potentially, higher recoveries could have been observed at TEMP > 80 °C, but were not explored because of concerns regarding the impact of solvent boiling on SPME fiber efficiency (*37*). We also observed a 10-fold decrease in [$^2H_2$]-IBMP peak area at 30 °C compared to the peak area obtained from aqueous buffer, but the decrease was only a factor of 2 at 80 °C extraction temperature (data not shown). Grape homogenate has been reported previously to reduce recovery of volatiles by HS-SPME (*38*), and tomato fruit homogenate has been reported to reduce SPME recovery of volatiles by 2−12-fold, likely because of noncovalent interactions between matrix components and volatile compounds (*39*). Whole berry homogenates are expected to have moderate levels of polyphenols, which have been reported to decrease the volatility of aromatic compounds like IBMP via $\pi-\pi$ stacking (*40*, *41*). Additionally, the lipid content of these samples is higher due to maceration of the seeds. Thus, unlike in juice studies, high extraction temperatures appear necessary to disrupt matrix−IBMP interactions and increase partitioning into the SPME stationary phase. This is also dissimilar to wine, where ethanol will decrease response of MPs at TEMP > 45 °C, likely due to competition for binding sites on the SPME fiber (*27*, *29*, *30*).

The [$^2H_2$]-IBMP signal increased with increasing extraction time (EXT), and equilibrium was not reached at all TEMP even at EXT = 140 min. Sala et al. (*32*) had previously reported that 4 h was required to achieve equilibrium during SPME extraction of MP from juice. However, Ryan et al. (*30*) reported that good

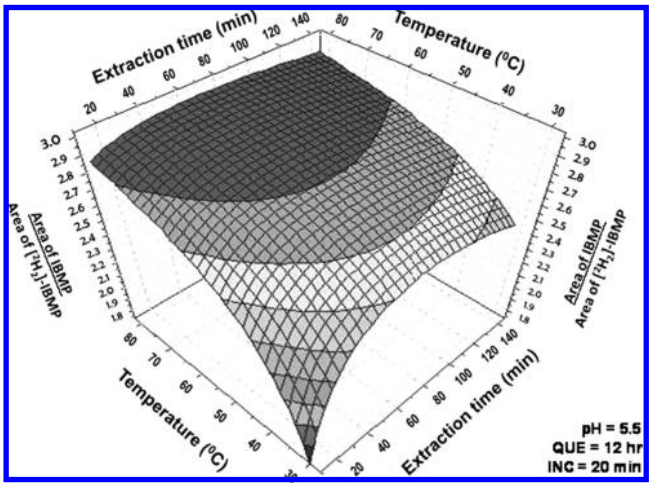

Figure 5. Response surface plot of TEMP versus EXT with the ratio of native IBMP to the internal standard, [124]/[126] ratio, as the response.

accuracy could be achieved in MP analysis of wine by 30 min HS-SPME extractions by using an isotopically labeled standard. Thus, assuming that sufficient time is available for endogenous MP to equilibrate with the isotopically labeled standard on the SPME fiber, an accurate measurement can be obtained. Therefore, the IBMP to [$^2H_2$]-IBMP ratio is expected to eventually plateau with increasing extraction time, at which point an accurate measurement can be achieved.

To determine the effect of extraction conditions on accuracy, the ratio of IBMP to [$^2H_2$]-IBMP (m/z = [126]/[124]) was plotted (**Figure 5**). The ratio of IBMP/[$^2H_2$]-IBMP generally increases with increasing EXT and TEMP, but plateaus at a high TEMP (70−80 °C). At TEMP = 80 °C, the difference in IBMP/[$^2H_2$]-IBMP ratio between EXT = 10, 70, and 140 min is < 5% (**Figure 5**) even though the peak area increases 3-fold (**Figure 4**). At lower EXT temperatures, the apparent IBMP concentration is less than the true value. The apparent equilibration at TEMP = 30 °C between EXT = 70 and 140 min is due to the higher error observed at 30 °C (**Figure 3**). IBMP is located in the exocarp (*31*), and the diffusion rate of moderately polar grape components from skin cells into juice (e.g., anthocyanins) is known to increase with increasing temperature and skin contact time (*42*). Taken together, the results in **Figures 4** and **5** indicate that there is a faster, temperature-dependent extraction of IBMP into the extracellular matrix, followed by a slower, temperature dependent extraction of IBMP onto the SPME fiber. While sensitivity is increased by increasingly higher EXT, acceptable accuracy is observed with EXT = 10 min as long as TEMP = 80 °C. For our work, we observed the optimal trade-off for sensitivity and extraction time at EXT = 30 min.

The other parameters (PH, INC, and QUE) were determined to have no significant effect on either [$^2H_2$]-IBMP peak area or method accuracy. The impact of pH on IBMP recovery from must has not previously been reported, but several groups have observed no impact of this parameter on MP recovery from wine (*26*, *27*, *30*). Queue times, QUE, and incubation time, INC, also had no impact on method sensitivity or accuracy.

Limit of Detection. Limits of detection were determined by preparing standard additions into either preveraison or harvest-ripe berry homogenate. Regression curves were generated for plots of m/z = [124]/[126] vs [IBMP addition] or m/z = [137]/[124] vs [IPMP addition], and regression parameters (slope, intercept, R^2) for the different experiments are shown in **Table 3**. The observed linearity was excellent, with all regression coefficients, R^2, > 0.99.

Table 3. Regression Parameters (Slope, Intercept, R^2) for Standard Addition of IBMP and IPMP into Either Pre- or Postveraison Berry Matrices[a]

analyte (matrix)	slope of regression	regression intercept	R^2
IBMP (preveraison berries)	0.145	0.648	0.993
IBMP (harvest berries)	0.150	0.450	0.997
IPMP (preveraison berries)	0.219	0.166	0.990
IPMP (harvest berries)	0.234	0.124	0.996

[a] Regression curves were generated for plots of m/z = [124]/[126] vs [IBMP addition] and m/z = [137]/[126] vs [IPMP addition]. Six standard addition levels were used, ranging from 0.5 to 200 pg/g for preveraison berries and from 0 to 200 pg/g for postveraison berries. Three replicates were performed at each standard addition level, for a total of 18 standard addition experiments per analyte and berry matrix.

Table 4. Coefficients of Variation (% CV) at Varying Levels of IBMP[a] and IPMP[a] Resulting from Standard Addition

	preveraison berries		harvest-ripe berries	
spike level	pg/g of IBMP (% CV)	pg/g of IPMP (% CV)	pg/g of IBMP (% CV)	pg/g of IPMP (% CV)
1	4.7 (17%)	0.7 (50%)	3.5 (9%)	1.0 (11%)
2	5.2 (3%)	1.2 (38%)	5.0 (10%)	2.5 (23%)
3	6.7 (3%)	2.7 (20%)	8.0 (7%)	5.5 (16%)
4	14.7 (3%)	10.7 (3%)	53.0 (1%)	50.5 (14%)
5	54.7 (4%)	50.7 (12%)	103 (3%)	100.5 (6%)
6	204.7 (8%)	200.7 (5%)	203 (2%)	200.5 (3%)
σ_i, pg/g [b]	0.2	0.6	0.4	0.3
LOD, pg/g [b]	0.6	1.8	1.2	0.9

[a] IBMP and IPMP concentrations are a sum of endogenous MP levels and spiking addition. % CV is the relative standard deviation of replicate measurements (n = 3) at the stated concentration. [b] σ_i = signal independent background noise, LOD = limit of detection.

The robustness of the method in the presence of different berry matrices was of initial concern, as preveraison berries have a notably different constitution than ripe berries. However, the responses, i.e. slopes of the regression curves, differ by < 10% in the preveraison and harvest-ripe berry matrices for IBMP (0.145 and 0.150, respectively) and IPMP (0.219 and 0.234, respectively). The similarity in slopes for IBMP between different matrices is unsurprising since a deuterated IBMP standard was used. However, the similarity of slopes for IPMP in the different matrices is more surprising. In HS-SPME analyses of wine, increasing ethanol content causes a large decrease in IPMP compared to IBMP response (*24*). However, it appears that changes in berry matrix during ripening (acids, cellulose, sugars) do not have a differential effect on response of IBMP and IPMP.

Through the standard addition method, we determined that the Pixie cultivar used in this study possessed low levels of both MPs. IBMP was present in preveraison berries at 4.7 pg/g and in harvest−ripe berries at 3 pg/g. IPMP was present in preveraison berries at 0.7 pg/g and in harvest−ripe berries at 0.5 pg/g. Coefficients of variance (% CV) for each standard addition level, analyzed within a single day, are reported in **Table 4**, with the spiked levels corrected by adding the endogenous MP concentration. Signal independent noise (σ_i) was calculated by regression analysis of total variance vs MP concentration (Pallesen's method) with a $1/X$ weighting factor. The limit of detection was calculated as ($3\sigma_i$) The LOD for IBMP and IPMP were 0.6 pg/g and 1.8 pg/g in preveraison berries, and 1.2 pg/g and 0.9 pg/g in harvest-ripe berries. The LOD range (0.6−1.8 pg/g) is slightly higher than the 0.2 pg/g threshold reported by Sala et al. by HS-SPME−GC−NPD of MPs in must (*32*). However, in the previous report the authors defined their noise threshold based on the variance of the chromatographic baseline. In our current report, we use the more stringent definition of calculating noise based on the error associated with the entire analysis, which gives a more realistic evaluation of the protocol capabilities.

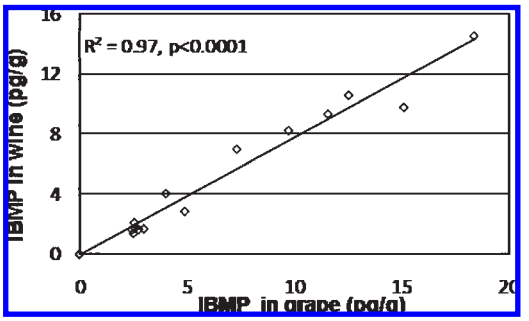

Figure 6. Correlation of IBMP concentration in Cabernet Franc whole berries from 16 sites in New York State compared to finished wines. Each data point represents the mean of two analytical replicates.

Correlation of IBMP in Whole Berries to Wine. Cabernet Franc fruit was collected from 16 vineyard sites and vinified in separate lots. The sites were selected to represent different environmental conditions and cultural practices, and resulted in a wide range of fruit chemistries. Classic juice parameters (Brix, TA, and pH) and average fresh berry weight were measured. Total soluble solids ranged from 18.8 to 23.0 °Brix (median = 20.6 °Brix), and pH ranged from 3.28 to 3.97 (median = 3.41). TA values were 7.8 to 11.6 g/L (median = 10.1 g/L), and berry weight averages ranged from 1.31 to 1.78 g. The fruit was vinified under identical conditions at a pilot-scale winery. Following crushing, but prior to inoculation, free-run juice samples were collected. IBMP and IPMP were quantified in whole berries from the 16 vineyard sites using the previously optimized methodology. Quantification was also performed on free run juices and wine samples under identical conditions to berry homogenates, with the exception that the extraction temperature was reduced to 40 °C for wines. IBMP concentrations in whole berries ranged from undetectable to 18.4 pg/g, and from undetectable to 14.5 pg/g in wines.

A plot of IBMP content in wines vs the initial grapes (**Figure 6**) shows a very high correlation ($R^2 = 0.97$, $p < 0.0001$), demonstrating that the method is appropriate for predicting IBMP in finished wines based on levels in grapes. Previous reports have shown poor correlations between IBMP concentrations in juice and the resulting wines (*23*, *24*, *43*). In our work, we observed minimal extraction of IBMP concentrations into the free run juice (n.d. - 1 pg/g). The optimization experiments on must indicated that high temperatures or prolonged skin contact times are necessary to facilitate diffusion of IBMP from the skins into the juice. Therefore, previous difficulties encountered in correlating IBMP levels in juice to IBMP levels in wine are likely due to incomplete and inconsistent extraction.

The ratio of the IBMP concentration in wine (pg/g) vs the IBMP concentration in grapes (pg/g) varied from 0.56 to 1.01 (mean = 0.77). However, this ratio does not account for the change in density that occurs during fermentation, which results in a 10−12% increase in concentration of remaining components. Following this correction, the observed concentration of IBMP in wines was 67 ± 13% (range = 50−91%) of the total berry IBMP. No significant correlation was observed between this extraction efficiency metric and Brix, berry pH, TA, average berry size, initial berry MP content, or wine MP content (**Table 5**).

The fate of IBMP that is not retained during fermentation is not known, nor are the factors that determine the extraction efficiency from berries into wine. The reasons for these differences are worthy of further study. However, the observed range in

Table 5. Correlation of IBMP Extraction Efficiency, with Berry Brix, pH, TA, Weight, Initial Berry IBMP Content, and Final Wine IBMP Content[a]

correlation	regression coefficients (R^2)	P value
Brix	0.044	0.489
pH	0.006	0.792
TA (g/L)	0.100	0.268
weight (g)	0.008	0.752
IBMP in berries (pg/g)	0.082	0.322
IBMP in wines (pg/g)	0.196	0.113

[a] Extraction efficiency was calculated as the ratio of wine IBMP to berry IBMP, corrected by the loss of mass during fermentation.

extraction efficiency is less than a factor of 2, compared to an order of magnitude range in berry IBMP concentration. Thus, the whole berry homogenate approach still results in useful predictions of final IBMP, and underscores the importance of cultural practices in controlling MP levels in finished wines. Further work is necessary to determine if the extraction efficiency varies appreciably with site, vintage, vinification practice, or cultivar.

IPMP was not detectable (<0.6 pg/g) in any berry sample. Existing data on the presence of IPMP in the Bordeaux cultivars uncontaminated by ladybeetle taint are contradictory, with some studies reporting 5 pg/g or greater in the majority of wines studied (*12*, *44*−*46*), while other studies reporting that IPMP is undetectable in nearly all wines (*4*, *18*, *28*, *35*). We note that the methodologies employed in studies that do not observe IPMP use more selective GC−MS methodologies, i.e. comprehensive 2-D GC, multidimensional GC, GC−MS/MS, or else more thorough sample cleanup (distillation, cation-exchange prior to liquid−liquid extraction). In our work, IPMP coelutes with several other interfering compounds in the first GC dimension on a 5% phenyl column even when viewing a selected ion (**Figure 1**), but these interferences can be resolved by GC×GC. Therefore, it is possible that some reports relying on 1-D GC with minimal sample cleanup may have unrecognized interferences. However, it is also possible that IPMP concentrations differ considerably between regions. This matter deserves closer consideration in future studies.

In summary, we have developed a rapid, optimized procedure for quantification of IBMP and IPMP in whole berries based on bead milling of small sample sizes followed by high temperature HS-SPME extraction. The method results in complete extraction of MPs into the extracellular matrix and accurately predicts MP levels in finished wines. Using conventional red winemaking practices, the observed concentration of IBMP in wines was $67 \pm 13\%$ of berry IBMP, although the factors responsible for the observed range in extraction efficiencies are not understood. One final advantage of the bead-milling approach is that it eliminates precrushing and extraction of the juice. Potentially, the bead-milling methodology could be adopted for measurement of MPs at a single berry level, albeit with a loss of sensitivity. This analytical approach would allow measurement of the distribution of MPs within a cluster, a currently unattainable goal.

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Supporting Information Available: Table S1 providing a full list of combinatory values of PH, TEMP, QUE, INC and EXT for each run during HS-SPME optimization. Values were generated by a center composite face-centered statistical model. This material is available free of charge via the Internet at http://pubs.acs.org.

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