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

Imelda Ryona,[†] Sophie Leclerc,[‡] and Gavin L. Sacks*,[†]

[†]Department of Food Science and Technology, Cornell University, 630 West North Street, Geneva, New York, and [‡]Institut national supérieur des sciences agronomiques, de l'alimentation et de l'environnement, AgroSup Dijon, 26 Boulevard Docteur Petitjean, 21000 Dijon, France

Environmental factors affecting degradation of 3-isobutyl-2-methoxypyrazine (IBMP, "green pepper aroma") in wine grapes (*V. vinifera*) are widely studied, but the degradation pathway is not defined. We hypothesized that IBMP is demethylated to 3-isobutyl-2-hydroxypyrazine (IBHP) during fruit maturation effectively reversing the final putative step of IBMP biosynthesis. A quantification method for IBHP was developed using solid-phase extraction coupled to one- or two-dimensional gas chromatography−mass spectrometry with a recovery of ca. 80%. IBMP and IBHP in bell peppers (*Capsicum annuum*) and *V. vinifera* (cv. 'Cabernet Franc', 'Riesling', 'Pinot noir') were then measured at different maturities. IBMP and IBHP were inversely correlated in both bell peppers (R^2 = 0.958) and Cabernet Franc grapes (R^2 = 0.998) over a range of maturities. In bell peppers, we observed a significant decline in IBMP (125 to 15 ng/mL) and increase in IBHP (undetectable to 42 ng/mL) during ripening. In grapes, all cultivars had comparable IBHP concentrations preveraison (64 to 88 pg/mL) but differed in IBHP concentration by 2 orders of magnitude at the final sampling point (undetectable to 235 pg/mL). Higher preveraison IBMP was correlated with higher final IBHP across the three grape cultivars, with the order Cabernet Franc > Riesling > Pinot noir for both IBMP and IBHP. Acid hydrolysis resulted in a significant increase (33%) in IBHP in Cabernet Franc, indicating that IBHP exists partially in a bound form in grapes.

KEYWORDS: Alkylmethoxypyrazine; hydroxypyrazine; GC×GC−TOF-MS; grape maturation

INTRODUCTION

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

*Author to whom correspondence should be addressed. E-mail: gls9@cornell.edu. Tel: (315) 787-2458. Fax: (315) 787-2397.

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

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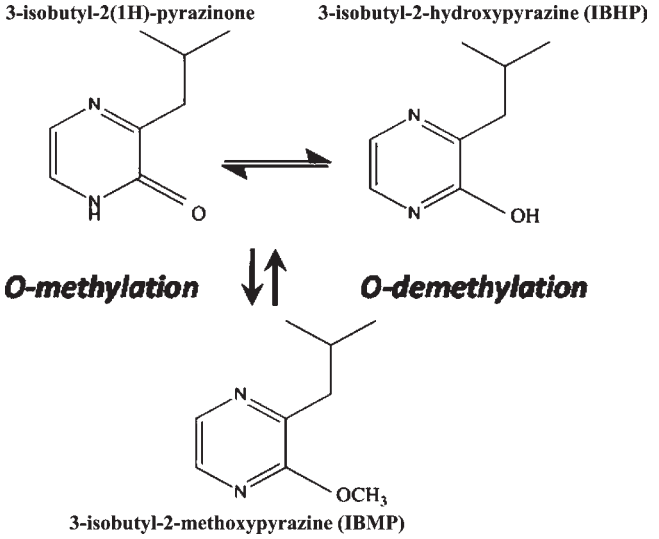

Figure 1. Putative biosynthesis and degradation pathways of IBMP/IBHP through O-methylation or O-demethylation reactions. IBHP coexists with its tautomer, 3-isobutyl-2(1*H*)-pyrazinone.

methyltransferase capable of converting 3-isobutyl-2-hydroxypyrazine (IBHP) to IBMP has been isolated from Cabernet Sauvignon wine grapes (*17*). Based on this work, two putative O-methyltransferases (*VvOMT1* and *VvOMT2*) were cloned and shown to be capable of forming MPs via *O*-methylation of HP precursors (*18*). Regardless of the mechanism, the putative degradation product(s) of MPs in plants have not been reported in the literature. In rats, IBMP is reportedly metabolized to IBHP and IBHP glycosides following ingestion (*19*). The demethylation of IBMP to IBHP effectively reverses the final putative step in IBMP synthesis. We hypothesized that a similar pathway in which MPs are degraded to their corresponding HPs may occur in plants during fruit ripening (**Figure 1**). If this pathway exists, then an inverse quantitative relationship should be apparent between MPs and HPs during ripening. The presence of IBHP and 3-isopropyl-2-hydroxypyrazine (IPHP) has been previously reported in immature wine grapes (*13*), although not in bell peppers. The relationship of HP and MP during fruit maturation has not been studied. In this report, we demonstrate that IBMP and IBHP are inversely correlated during ripening in both wine grapes and in bell peppers.

MATERIALS AND METHODS

Chemical Reagents and Standards. Dichloromethane (DCM), methanol, pentane, ethyl acetate, citric acid, ascorbic acid and sodium hydroxide were purchased from Fischer Scientific(Pittsburgh, PA). DDI water was obtained from a Milli-Q purification system Millipore (Billerica, MA). Aroma standard, 2-*sec*-butylphenol, was purchased from Sigma Aldrich (Allentown, PA). 3-Isobutyl-2-hydroxypyrazine (IBHP) was purchased from Manchester Organics Ltd. (97%, Sutton Weaver, U.K.). 3-Isopropyl-2-hydroxypyrazine (IPHP) and 3-*sec*-butyl-2-hydroxypyrazine (sBHP) were synthesized in our laboratory by condensation of either L-valinamide·HCl (97%, Sigma-Aldrich, St. Louis, MO) or L-isoleucinamide·HCl (98%, TCI America, Portland, OR) respectively with glyoxal sodium bisulfate hydrate (Sigma-Aldrich) under alkaline conditions as described by Seifert et al. (*16*). The synthesized products were confirmed by comparison of MS spectra to those in earlier reports and the purity of products was checked by GC−MS.

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

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

Isolation of Free and Bound HPs from Juices. Due to the polarity of HPs, the SPE method of Ibarz et al. 2006 (*20*) for extraction of glycosides was adopted for extraction of these compounds. All SPE were performed on a Varian 24-cartridge positive pressure manifold (Palo Alto, CA). SPE sorbent conditioning was performed by 2.5 mL of dichloromethane, 2.5 mL of methanol, and 5 mL of H_2O per 100 mg of sorbent. Bell pepper juice (50 mL) was percolated through a 3 mL cartridge packed with 200 mg of LiChrolut EN (Merck, Darmstadt, Germany). For grape samples, 1300 mg of LiChrolut EN sorbent was manually packed into a 12 mL cartridge, and 7 cartridges were used for extraction of 1000 mL of grape juice. Extraction of both bell pepper and grape samples were carried out in duplicate. After sample loading, the sorbent bed was washed with 2.5 mL of H_2O and 2.5 mL of pentane:DCM (2:1 v/v) per 100 mg of sorbent. Prior to elution, the bed was dried under pressure (25 psi, N_2) for 20 min. Subsequently, the targeted fraction was eluted with 4 mL (for bell pepper samples) or 25 mL (for grape samples) of ethyl acetate. For bell pepper samples, 20 μL of a 2-*sec*-butylphenol internal standard (50 mg/L in ethanol) was added to the 4 mL of eluent and concentrated to 0.3 mL with a continuous N_2 gas flow prior to GC−TOF-MS analysis. For grape samples, the 175 mL (25 mL × 7 cartridges) of eluent was concentrated to ca. 5 mL at 40 °C on a Buchi R-210 Rotavapor. Then, it was evaporated to dryness under N_2, reconstituted in 20 mL of 0.2 M citric acid buffer adjusted to pH 2.5, and spiked with 20 μL of a 2-*sec*-butylphenol standard (50 mg/L in ethanol). The reconstitute was then subjected to a second SPE (200 mg LiChrolut EN cartridge) preconditioned with 5 mL of DCM, 5 mL of methanol, and 5 mL of H_2O. After loading, the column was dried under N_2 (20 min, 25 psi) and HPs were eluted with 2.8 mL of ethyl acetate. The eluent was concentrated to ca. 0.3 mL for GC×GC−TOF-MS analysis.

Analysis of Bound HPs from Juices. To determine if bound, acid-hydrolyzable HPs were present, an additional step was introduced following the initial SPE isolation step described in the previous section. Red pepper and Cabernet Franc juice samples were prepared separately from the previous study on free HPs. Following the initial SPE, the ethyl acetate fractions were evaporated to dryness and reconstituted with 10 and 20 mL of citric acid buffer for the red pepper and Cabernet Franc samples, respectively. The buffer solution was heated in a 100 °C water bath for 1 h in an encapsulated vial under a N_2-filled headspace, as described by Ibarz et al. (*20*). Twenty microliters of 2-*sec*-butylphenol (50 mg/L) was then added to the hydrosylate prior to a second SPE isolation on a 200 mg LiChrolut EN cartridge preconditioned according to the above procedure.

The cartridge was dried under N_2 (25 psi, 20 min) and eluted with 2.8 mL of ethyl acetate, which was subsequently concentrated under N_2 to ca. 0.3 mL prior to GC(×GC)−TOF-MS analysis. Additionally, we evaluated if free or acid-hydrolyzable bound forms of IBHP were not retained during loading or were lost during the water wash prior to elution. To evaluate this, the water wash fraction was combined with the unretained red pepper juice fraction, and a portion was treated by the acid hydrolysis method described above. The hydrolyzed and nonhydrolyzed juices were then re-extracted by the SPE method described in the previous method.

Quantification of HPs in SPE Extracts by GC(×GC)−TOF-MS. Quantification of HPs in extracts was performed on a two-dimensional gas chromatography coupled to a time-of-flight mass spectrometer, GC×GC−TOF-MS (Pegasus 4D, LECO Corp., St. Joseph, MI). For bell pepper analysis, quantifications of HPs and MPs were performed in 1D with the modulator turned off. Grape samples were analyzed in 2D mode, GC×GC−TOF-MS. The first dimension column was a CP Wax 52CB (30 m × 0.25 mm × 0.25 μm, Varian, Walnut Creek, CA), and the second dimension column was a VF-17 ms (2 m × 0.1 mm × 0.2 μm, Varian). A programmable temperature vaporization (PTV) injector was used, with an initial injector temperature of 50 °C held for 0.5 min and then ramped at 200 °C/min to 250 °C. The injector was operated in pulsed splitless mode, where the injector pressure was held at 45 psi for 2.5 min. The purge was opened after 3 min. Helium was used as a carrier gas at a flow rate of 1 mL/min. The injection size was 1 μL. The temperature program was as follows: initial hold for 5 min at 55 °C, followed by a 10 °C/min ramp to 100 °C; then, 3 °C/min to 240 °C, 30 min hold. The MS transfer line temperature was 260 °C. The TOF-MS was operated in EI mode with ionization energy of 70 eV. The electron multiplier was set to 1700 V. MS data from m/z = 20−400 was stored at 5 and 150 Hz for 1D and 2D analyses respectively. For GC×GC analysis, the modulation period was set for 3 s with a 0.75 s hot pulse time. The secondary oven temperature offset and modulation temperature offset were set for +20 °C. Data processing was carried out by the LECO ChromaTOF software. The qualifier ions were as follows: for IBHP, m/z 110 (100), 137 (24), 81 (19), 152 (9); for IPHP, m/z 123 (100), 95 (71), 110 (52), 138 (37); and sBHP were m/z 124 (100), 110 (65), 95 (61), 137 (39). The quantifier ion for all HPs was m/z 110. The qualifier ions for the internal standard 2-*sec*-butylphenol were m/z 121 (100), 150 (25), 77 (18), 103 (17), and the quantifier ion was m/z 150. The tolerance for the qualifier ions was ±20%.

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

$$\text{recovery of IBHP} = \frac{\text{spiked juice matrix IBHP} - \text{native IBHP}}{\text{spiked reference IBHP} - \text{native IBHP}} \times 100\%$$

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

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

Extraction and Quantification of IBHP in Water-Insoluble Fraction of Bell Pepper. Red bell pepper was macerated as described in the previous section and filtered through cheesecloth. The filtered juice (120 mL) was centrifuged for 30 min at 10,000 rpm and 5 °C. The water-insoluble material retained on the cheesecloth was collected. Subsequently, the insolubles (13 g) were suspended in 26 g of 100% methanol and incubated at 25 °C for 2 h with constant agitation at 200 rpm. The mixture was then centrifuged using the same conditions as above. The supernatant was filtered and evaporated by Rotavapor at 40 °C for 15 min. One half of the juice fraction (60 mL) was used to reconstitute the extract. IBHP in the juice + insoluble extract was then analyzed by SPE followed by GC−TOF-MS and compared to IBHP in the other half of the juice sample with no added extract.

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

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

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

RESULTS AND DISCUSSION

SPE Method Optimization. LiChrolut EN SPE has been previously used for recovery of polar compounds like furaneol and glycosides (*20*, *22*), and these methods were adopted for isolation of HPs. The method utilizes H_2O for washing the cartridge prior to elution of the HP analytes. Since the HPs are moderately polar, we were concerned they would be eluted prematurely during the wash step. To evaluate this hypothesis, the unretained fraction following percolation of red pepper juice through the SPE column was combined with the water wash fraction and treated with and without hydrolysis. The hydrolyzed and non-hydrolyzed samples were then repercolated through a new pre-conditioned SPE column. Results showed no detectable IBHP in this aqueous fraction for both hydrolyzed and nonhydrolyzed samples (data not shown). DCM, methanol and ethyl acetate were evaluated for their ability to elute IBHP from the SPE cartridge. Results showed a poor recovery of IBHP (< 10%) using dichloromethane. Methanol showed a modestly improved recovery of IBHP compared to ethyl acetate (~10%). However, the use of methanol required an additional step for removal of H_2O prior to GC analysis and also resulted in more peaks and lower signal-to-noise ratio in the chromatography. Thus, ethyl

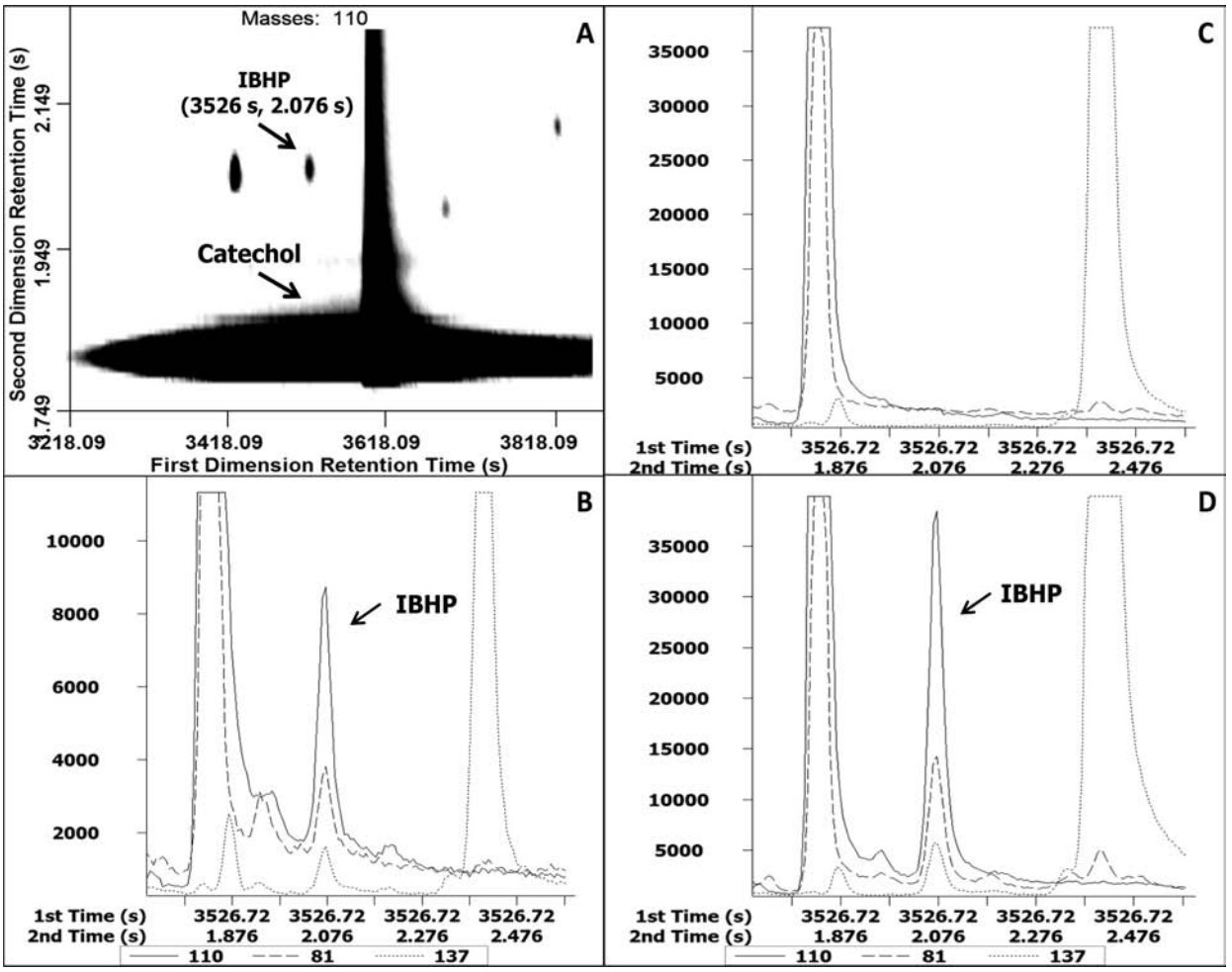

Figure 2. (**A**) Contour plot displaying *m*/*z* 110 ion and (**B**) unfolded GC×GC chromatogram displaying *m*/*z* 110, 81, and 137 illustrating separation of IBHP from an interference (library identification as catechol) in postveraison Cabernet Franc sample. Citric acid buffer with Pixie grape extract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

acetate was chosen as the elution solvent in this study. To confirm that sufficient ethyl acetate was utilized during SPE, a 2.8 mL volume of ethyl acetate was used to re-elute a 200 mg LiChrolut EN SPE column that had been previously eluted with ethyl acetate. No IBHP was detected in the second elution. Although direct immersion solid-phase microextraction (SPME) was not attempted, the feasibility of headspace-SPME with both polar and nonpolar fibers was evaluated at 40 °C for 30 min. No IBHP peak was visible using HS-SPME, likely because of the low volatility of IBHP. This may explain why IBHP was not observed in a recent paper that profiled 16 *Capsicum* species, as the authors in this study used HS-SPME for extraction of volatile constituents (*23*).

Detection of HPs by GC(×GC)-TOF-MS in Juices. IBHP was readily detectable in green and red pepper juice using 1-D GC−TOF-MS and its identity confirmed by comparison of retention time and mass spectra to an authentic standard. To our knowledge, this is the first confirmation of IBHP in a plant species other than *V. vinifera*. The concentration of IBHP was > 2 orders of magnitude higher in our pepper samples than in our grape samples. During sample preparation, a 20-fold greater volume of grape juice (1000 mL) than pepper juice was used to facilitate IBHP detection. However, a concurrent increase in interfering compounds was observed, such that no IBHP could be observed with 1-D GC−TOF-MS using standard polar and nonpolar columns. Resolution of IBHP from other interferences could be achieved by 2-D GC×GC−TOF-MS using a strong polar X medium polar column set (CP Wax 52 CB X VF-17 ms). **Figures 2A** and **2B** show representative contour and unfolded GC×GC chromatogram plots of IBHP, respectively, in postveraison Cabernet Franc. **Figure 2B** depicts one of the modulated slices in the unfolded chromatogram, and clearly shows both quantifier ion and qualifier ions of IBHP (first t_R 3526 s, second t_R 2.076 s, and a first dimension Kovats Index = 2713). Comparison of a citric acid buffer + Pixie grape extract with no detectable IBHP (**Figure 2C**) to the same sample spiked to 50 ng/mL IBHP (**Figure 2D**) confirmed the identification of IBHP in grape samples. GC×GC−TOF-MS permitted resolution of IBHP from a prominent interference (*m*/*z* 110, 64, and 81, second t_R = 1.7 s), tentatively identified by NIST library search as catechol. Since this interference was not observed in bell pepper matrix, the catechol peak was thought to be derived from thermal degradation of anthocyanins or other polyphenolics. Other column sets were unsuccessful in resolving IBHP. The use of a nonpolar VF-5 (2 m × 0.1 mm i.d. × 0.4 μm) as the second dimension column with the CP Wax 52CB as the first column was not able to resolve the catechol interference. A standard nonpolar × polar column set, VF-5 X VF-17 ms, failed to separate IBHP from a different interference (data not shown).

The other HPs, sBHP and IPHP, were not detectable in red pepper or grape juice samples, even when using GC×GC−TOF-MS for pepper samples. We had expected the ratios of different

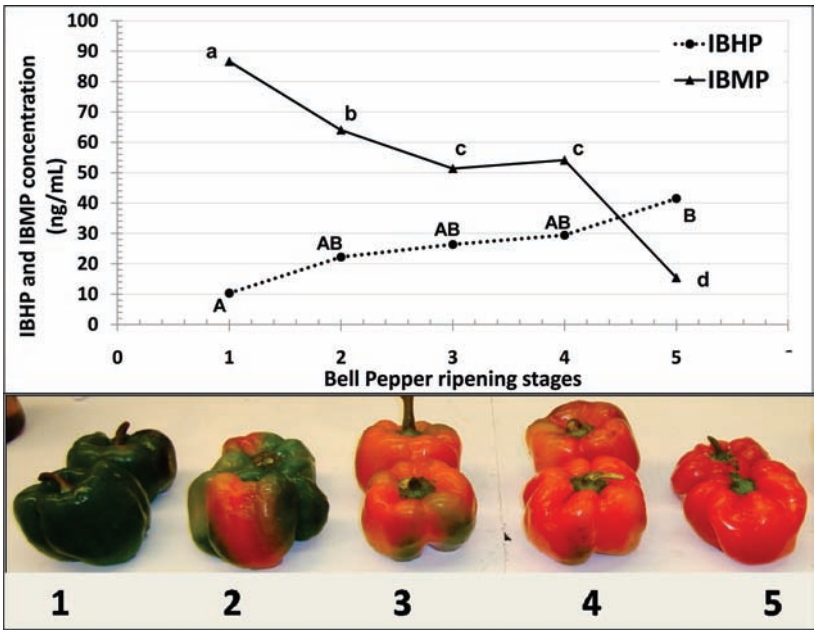

Figure 3. IBMP and IBHP concentrations, ng/mL, in bell pepper during ripening at 5 maturity stages. Different letters indicate a statistically significant difference at $p < 0.05$.

HP species to be proportional to the ratios of MP species. The ratio, IBMP:sBMP:IPMP, is reported to be approximately 100:1.5:1 in green peppers (*3*), 50:0.15:1 in red peppers (*3*), and 36:5:1 in wine grapes (*24*). With our current methodology, we may be below the detection thresholds for sBHP and IPHP. The detection thresholds were approximately 1 ng/mL in pepper (50 mL sample) based on thresholds for IBHP.

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

In model juice, calibration standards from 0 to 60 ng/mL IBHP resulted in a linear calibration curve (R^2 = 0.998). The IBHP detection threshold for the pepper extraction protocol was determined to be 1 ng/mL. For grapes, calibration standards were prepared from 0 to 200 ng/mL IBHP in the citric acid buffer, or effectively 0 to 4000 pg/mL in grapes. Good linearity was achieved (R^2 = 0.999), and the IBHP detection threshold in grapes was calculated to be 25 pg/mL by Pallesen's method following correction for recovery. Limits of detection for IBHP in grape juice were high as compared to reports on IBMP analysis for two reasons. First, the precision of our IBHP measurements was limited partially by its coelution with the tail of the catechol interference, even in the GC×GC mode. Second, the response factor was lower for the IBHP quantifier ion, such that the IBMP quantifier ion (m/z 124) had 6× higher signal compared to IBHP peak (m/z 110) at the same injected concentration.

IBMP versus IBHP Concentration in Bell Pepper. In this study, analysis of IBMP and IBHP was conducted on defrosted bell pepper. While tissue disruption caused by freezing is reported to change some volatiles in peppers and other plants, e.g. C6 aldehydes, IBMP is reported to be unaffected by enzymes released through tissue damage (*25*). Visual appearance was used to assay 5 different ripening stages (**Figure 3**), as reducing sugars did not prove to be a useful metric for ripening. Reducing sugars, glucose + fructose, ranged from 29.6 to 37.7 g/L in peppers, with a mean value of 34.8 g/L. The reducing sugar concentrations are 2-fold lower than those previously reported (*26*), and were not correlated with visual maturity, IBHP concentration, or IBMP concentration.

During ripening, IBMP decreased from 86.6 ng/mL (green, stage 1) to 15.4 ng/mL (red, stage 5) with intermediate concentrations observed in stages 2−4. The decrease in IBMP during bell pepper ripening has been previously reported (*27*). The IBMP concentrations in peppers measured in this study were higher than previously reported concentrations, 20 ng/g and 5.5 ng/g in green and red peppers, respectively (*3*). This discrepancy could be due to biological variation, e.g. different cultivar, or it may be due to differences in sample preparation, as the earlier report used purge-and-trap for extraction and may not have quantitatively recovered IBMP. During pepper ripening (**Figure 3**), there was a corresponding increase in IBHP from 10.3 ng/mL (green, stage 1) to 41.5 ng/mL (red, stage 5). Stage 1 had significantly lower IBHP than stage 5. Stages 2, 3 and 4, with a mixture of green and red color, demonstrated no significant difference in IBHP as compared to stage 1 or stage 5.

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

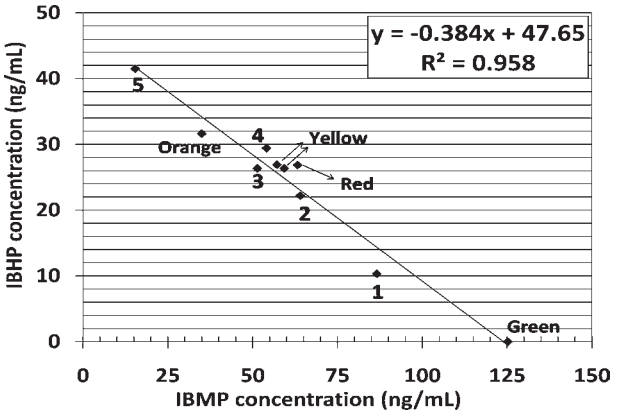

Figure 4. IBMP versus IBHP concentrations in bell pepper sampled from different sources and at different maturities. Numbered samples correspond to their maturity stage in **Figure 2**. Samples labeled with a color name were not part of the **Figure 2** maturity study, and the label refers to the color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

Table 1. IBMP, IBHP, and Total Soluble Solid Concentrations in V*itis vinifera* Grapes during Berry Maturation[a]

	IBHP (pg/mL)	IBMP (pg/mL)	total soluble solid (°Brix)
	Preveraison		
Cabernet Franc (Aug 14th)	68 ± 1	259 ± 6	5.3
Pinot noir (Aug 14th)	88 ± 10	11 ± 0	5.5
Riesling (Aug 14th)	64 ± 4	71 ± 7	5.4
	Postveraison		
Cabernet Franc (Sept 23rd)	223 ± 10	30 ± 1	18.6
Pinot noir (Sept 23rd)	<LOD	<LOD	18.4
Riesling	NA	NA	NA
	Harvest		
Cabernet Franc (Oct 13th)	235 ± 7	2 ± 0	20.6
Pinot noir	NA	NA	NA
Riesling (Oct 20th)	78 ± 9	<LOD	20.7

[a] <LOD means below limits of detection (25 pg/mL for IBHP, 1.2 pg/mL for IBMP). NA means samples not available for this study. All values of IBHP were quantified as free IBHP without hydrolysis treatment.

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

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

IBMP and IBHP in *Vitis vinifera* Grapes. Cabernet Franc, Pinot noir, and Riesling were collected at various maturity stages and analyzed for IBMP and IBHP (**Table 1**). The preveraison concentrations of IBMP were as follows: Cabernet Franc (259 pg/mL), Riesling (71 pg/mL) and Pinot noir (11 pg/mL). During maturation, IBMP decreased to 30 pg/mL and then to 2 pg/mL in Cabernet Franc, and to below the limit of detection (< 1.2 pg/mL) (*8*) in Riesling and Pinot noir. The order, Cabernet > Riesling > Pinot, is in concordance with previous reports (*24*, *29*). Similar to bell pepper, we observed a significant increase in IBHP during maturation of grapes. IBHP concentration at the last sampling point for each cultivar was significantly different among all cultivars. The same order for harvest IBHP was observed for preveraison IBMP, with the highest IBHP observed in Cabernet Franc (235 pg/mL), followed by Riesling (78 pg/mL), and undetectable concentrations in Pinot noir. Interestingly, we observed comparable concentrations of IBHP in all cultivars preveraison (64−88 pg/mL), with the highest concentrations in the non-MP accumulating Pinot noir. The final step of IBMP synthesis is proposed to be O-methylation of IBHP to form IBMP, and a previous study demonstrated a positive correlation (R^2 = 0.779) of IBMP versus IBHP in 8 cultivars collected at 40 days postbloom (*13*). However, the authors also reported that preveraison Pinot noir, Riesling, and Cabernet Sauvignon had comparable concentrations of IBHP (ca. 3 nmol/kg fresh weight, or ca. 500 pg/mL), despite differing by over an order of magnitude in IBMP. Therefore, the poor correlation we observed between preveraison IBHP and IBMP for the three cultivars selected in our study was not unexpected. However, the concentrations of IBHP in our study were a factor of 10 lower than those previously reported by Hashizume et al. Similarly, up to 200 pg/L IPHP were detected in preveraison Riesling and Pinot noir in the previous study, but no IPHP was detected in our current study. The reason for the quantitative discrepancy between these studies is not clear.

Presence of Acid-Hydrolyzable "Bound" IBHP in Grapes and Peppers. Previous work on the metabolism of IBMP in rats demonstrated that the IBMP was demethylated to IBHP and then partially glycosylated (*19*). Because other aromatic alcohols are reported to be glycosylated postveraison in grape berries, e.g. guaiacol and 4-methylguaiacol associated with "smoke taint"(*30*), we hypothesized that IBHP may exist in glycosylated form in fruit. Following the first SPE, the extract was hydrolyzed and the hydrolysate re-extracted via a second SPE. The second SPE step was included on both "free" and "total" grape analyses to provide a similar extraction protocol for both hydrolyzed and nonhydrolyzed samples. To determine the proportion of IBHP in free and bound forms, IBHP in hydrolyzed and nonhydrolyzed samples was measured in duplicates of 50 mL red bell pepper and 1000 mL postveraison Cabernet Franc grapes. We observed a significant increase ($p < 0.05$, one-tailed t test) in the grape samples resulting from hydrolysis (143 ± 10 pg/mL hydrolysis vs 95 ± 15 pg/mL nonhydrolysis) and no significant difference in red pepper (58 ± 4 ng/mL hydrolysis vs 53 ± 8 ng/mL nonhydrolysis) (**Figure 5**). Thus, a fraction of IBHP in grapes (33%) appears to exist in an acid-hydrolyzable bound form, potentially a glycoside. This distribution is similar to the excretion patterns of IBHP and its glycoside following IBMP metabolism observed in rats.

Based on these results, we were concerned that the IBHP peak observed for grape samples (**Table 1**) may be partially derived from thermal degradation of IBHP precursor forms. However, increasing the cool hold time of PTV injector following injection resulted in a 2-fold decrease in peak area for both the IBHP peak and the *sec*-butylphenol internal standard, resulting in no change in IBHP quantification. By comparison, the catechol interference decreased markedly under the lower injector temperature conditions, supporting the idea that the catechol peak is due to thermal degradation of polyphenols.

Evaluation of Hypothesis That IBMP Is Demethylated to IBHP during Fruit Maturation. We undertook this study with the

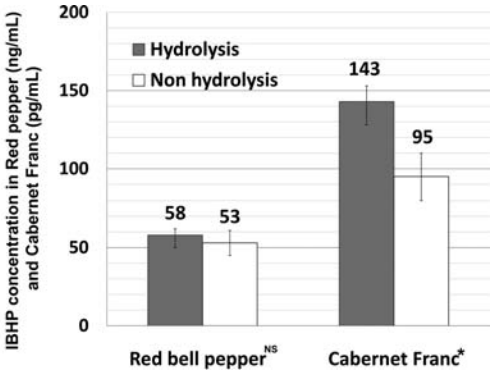

Figure 5. Hydrolyzed and nonhydrolyzed IBHP levels in red bell pepper and mature Cabernet Franc. NS indicates no statistically significant difference, and * indicates a statistically significant difference (one-tailed *t* test, $p < 0.05$). The error bar reflects standard deviation of the duplicates.

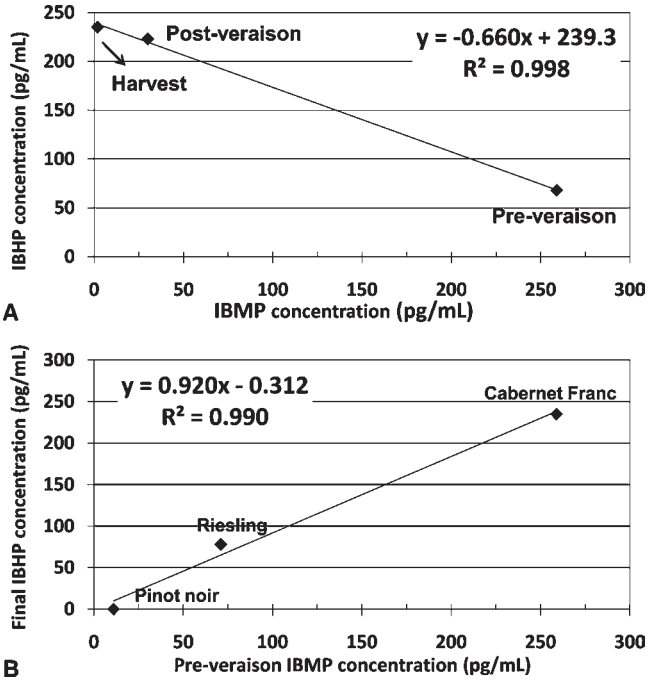

Figure 6. (**A**) IBMP versus IBHP concentrations in pre- and postveraison and harvest Cabernet Franc samples and (**B**) preveraison IBMP versus final IBHP concentrations in Cabernet Franc, Pinot noir and Riesling.

hypothesis that MPs are degraded to HPs and/or HP glycosides during fruit maturation. In rats, IBMP is reportedly metabolized to IBHP and IBHP glycosides following ingestion (*19*). If a similar phenomenon occurred in fruit, we would expect to see a quantitative increase in IBHP as IBMP concentrations decrease, which we observe. A plot of IBHP vs IBMP for Cabernet Franc yielded a strong inverse correlation, $R^2 = 0.998$ (**Figure 6A**). Assuming that IBHP was not further transformed, we would also expect to observe a strong correlation between preveraison IBMP and final IBHP. We observe a significant, positive correlation, $R^2 = 0.990$, between preveraison IBMP and final IBHP across the 3 cultivars we studied with the order Cabernet Franc > Riesling > Pinot noir for both analytes (**Figure 6B**). Although further studies are clearly necessary, these data are compatible with the MP-to-HP degradation hypothesis. The data also indicate that IBHP concentrations at harvest could be used as a proxy for the maximum IBMP achieved preveraison. Our hypothesis would also predict that the total IBHP + IBMP (moles per berry) in fruit should not change during ripening. Unfortunately, we did not measure berry weights and thus cannot account for any dilution caused by berry enlargement, nor did we measure bound IBHP in the samples used for the maturity study data shown in **Table 1**.

The bell pepper data did not support the IBMP degradation hypothesis as clearly. While a very strong correlation was observed between IBMP and IBHP during maturation (**Figure 4**), the slope of the best-fit line was 0.38, and no bound, acid-hydrolyzable IBHP was detectable. Therefore, if degradation of IBMP to IBHP does occur during pepper ripening, ca. 60% of the IBMP present in the green peppers cannot be accounted for in the ripened peppers (**Figure 3**). Potentially, the IBHP is further metabolized in peppers, or additional IBMP degradation pathways exist. A final possibility is that O-methyltransferase activity and consequent methylation of IBHP to IBMP decreases during ripening in both peppers and grapes; the increase in IBHP thus reflects a decrease in metabolic flux, resulting in a buildup of the IBHP intermediate. Labeled precursor studies, as well as more detailed time course studies, will be useful in refining these hypotheses.

In summary, we have demonstrated that IBHP concentrations increase proportionally to the decrease in IBMP in both ripening peppers and wine grapes. These results provide some evidence that MPs are degraded to HPs during fruit ripening, but future work is necessary to determine the enzymes responsible. O-Demethylation of other biochemical substrates has been reported in plants (*31*). However, since OMT activity is reported to decrease sharply after 4 weeks post flowering in grapes, we consider it unlikely that this enzyme is responsible for performing the reverse reaction (*18*). If our hypothesis is true, free IBHP at harvest could be used as a proxy for the preveraison maximum IBMP. This would be useful for viticultural studies interested in determining factors that affect IBMP accumulation preveraison, as it would decrease the number of sampling time points necessary. Assuming that IBHP persists through during fermentation, it may even allow for post hoc evaluation of maximum IBMP concentrations in the vineyard by measuring IBHP in finished wines. In the case of Cabernet Franc wine grapes, we can detect a significant increase in IBHP following acid hydrolysis, indicating that IBHP may also partially exist as a glycoside, although this tentative conclusion should be confirmed, i.e. by synthesis of a standard and identification by LC−MS/MS, as has been reported for IBMP (*7*). Further studies on HPs in grapes will be facilitated by improved analytical methodologies. The current protocol requires 1 L of juice and 2-D GC×GC to avoid coeluting interferences. The use of a more selective cleanup protocol and/or use of LC−MS/MS could be appropriate for reducing sample size and avoiding interferences.

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