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2-Methoxy-3-isobutylpyrazine in grape berries and its dependence on genotype

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ABSTRACT

2-Methoxy-3-isobutylpyrazine (MIBP) contributes a bell pepper aroma to many grape cultivars and has a reported aroma threshold of ~2 ng L^{-1} in water. The purpose of this study was twofold: (1) develop a procedure using headspace solid phase micro-extraction combined with GC–MS in the selected ion monitoring mode (HS-SPME-GC–MS-SIM) for analysis of MIBP in grape berries, and (2) determine the location of MIBP biosynthesis in grapevines by approach grafting clusters of *Vitis vinifera* L. cvs Cabernet Sauvignon and Muscat blanc onto each other. The soluble solids and pH of the grape juice/homogenate matrix from different grape berry developmental stages influenced the method precision; therefore, quantification via the method of standard addition was used. Using our developed method, the limit of detection (LOD) and limit of quantitation (LOQ) of MIBP were 0.1 ng L^{-1} and 2 ng L^{-1}, respectively, measured in a model juice and non-MIBP containing Chardonnay juice. Spiked recoveries averaged between 91% and 112% in Cabernet Sauvignon and Pinot noir homogenates and the overall relative standard deviation was less than 10%. The method was used to analyze MIBP in 29 grape cultivars and in fruit from clusters grafted to Cabernet Sauvignon or Muscat vines. Quantifiable levels were found only in Cabernet franc, Cabernet Sauvignon, Merlot, Sauvignon blanc and Semillon, providing information on the genetic connection for the occurrence of MIBP in grapes. No MIBP was detected in the berries of Muscat blanc clusters grafted onto Cabernet Sauvignon vines when sampled at fruit maturity. MIBP was detected in all berries of Cabernet Sauvignon regardless the graft configuration. The data indicate that MIBP or its precursors originate in the berry and its formation depends upon grape genotype.

1. Introduction

2-Methoxy-3-isobutylpyrazine (MIBP) (**1**), 2-methoxy-3-isopropylpyrazine (MIPP), and 2-methoxy-3-*sec*-butylpyrazine (MPs-B) were reported in Freon extracts of *Vitis vinifera* L. Sauvignon blanc fruit (Augustyn et al., 1982) and confirmed using gas chromatography–mass spectrometry (GC–MS) following distillation and extraction of wines (Harris et al., 1987). Of these three methoxypyrazines, MIBP (**1**) (Fig. 1), which has a bell pepper aroma, is considered the most important because of its very low aroma threshold (2 ng L^{-1} in water) (Buttery et al., 1969) and relatively high concentration in grapes and wines (e.g., Lacey et al., 1991). Augustyn et al. (1982) proposed that MIBP (**1**) was key to the characteristic 'asparagus-like, green, grassy, bell pepper-like' aroma of Sauvignon blanc wines, and they cited Bayonove et al. (1975) as having made a similar suggestion – that MIBP (**1**) was responsible for the characteristic 'green note' in Cabernet Sauvignon grapes and wines. Since that early work, methods for quantifying volatiles have improved considerably, and the importance of understanding factors that impact MIBP levels in both Sauvignon blanc and in Cabernet-type wines has increased. In Cabernet Sauvignon, a high MIBP (**1**) concentration in grapes may have a negative impact on wine aroma quality (Allen and Lacey, 1999).

Headspace solid phase microextraction (HS-SPME) combined with GC–MS is widely used for analysis of volatiles in food and beverage samples because it is rapid and easily automated (Ebeler, 2001; Pawliszyn, 1997). Chapman et al. (2004) developed a HS-SPME-GC–MS method for analysis of MIBP (**1**) in wines with an accuracy of >95%, relative standard deviation (RSD) of <12%, and a limit of quantitation (LOQ) of 5 ng L^{-1}; however, the method was not validated in a grape or juice matrix. Similar approaches to quantifying MIBP (**1**) in grape berries have been described but they required long extraction times (Belancic and Agosin, 2007; Sala et al., 2000) or lacked sufficient sensitivity (Hartmann et al., 2002) for our application. Recently, Ryona et al. (2008, 2009) measured MIBP (**1**) in pulverized Cabernet franc grapes using HS-SPME combined with two-dimensional comprehensive gas chromatography coupled to a time-of-flight mass spectrometer (GC × GC-TOF MS). The GC × GC analysis improved separation from matrix interferences (Ryan et al., 2005; Ryona et al., 2008, 2009), however, absolute recoveries for the method were not reported. Use of MS for detection allows stable isotope labeled internal standards to be used which can significantly improve the accuracy and

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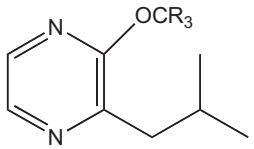

2-methoxy-3-isobutylpyrazine, R = H, (1)
2-($^2D_3$)-methoxy-3-isobutylpyrazine, R = ^{2}D (deuterium), (1a)

Fig. 1. Structure of 2-methoxy-3-isobutylpyrazine (MIBP) and the deuterated internal standard, 2-($^2D_3$)-methoxy-3-isobutylpyrazine (dMIBP), used in this study.

precision of the MIBP (**1**) analysis compared to use of a chemically similar, but not identical internal standard (Allen et al., 1994). However, for previous MIBP (**1**) analyses of grape berries, the internal standard was added after the berries had been homogenized and diluted (Belancic and Agosin, 2007; Ryona et al., 2008, 2009), so analyte recovery losses during these steps could not be accounted for.

Here, we describe a HS-SPME-GC–MS method for MIBP (**1**) analysis in grapes; we evaluated the effects of different grape sample preparation conditions and the effects of grape composition (soluble solids and pH) on the accuracy and precision of the method. The method was then used to survey 29 cultivars for the presence of MIBP (**1**) in fruit and to evaluate whether it is translocated from leaves to fruit using the reciprocal grafting technique of Gholami et al. (1995) who demonstrated that monoterpenes are not translocated from leaves to berries.

2. Results

2.1. Grape sample preparation

During initial method validation, sample preparation methods were compared using either frozen or thawed whole berries and skins only from frozen or thawed berries. The peak area of the MIBP (**1**) quantification ion (m/z = 124) was used to compare the different sample preparation methods for their ability to release MIBP (**1**) from the grape berries and skins (data not shown). Measurable differences in its amount were not observed in the headspace of the supernatants obtained with the different sample preparation methods or fruit parts used. The amount of MIBP (**1**) in the pellet remaining after centrifuging whole frozen berries was approximately one half that found in the supernatant (data not shown). Due to the ease of sample preparation, frozen, whole berries were used for all subsequent analyses using the procedures described in Experimental.

2.2. Calibration and linearity

Standard curves were prepared in model juice matrices, in a Chardonnay juice matrix and in a Pinot noir homogenate (Table 1). The Chardonnay and Pinot noir did not contain any measurable levels of background MIBP. The coefficients of determination (R^2) for the linear relationship between MIBP (**1**) concentration and peak area ratio for the model juice, the Chardonnay juice and the Pinot noir homogenate were in excess of 0.95. Similar results were obtained with the Cabernet Sauvignon homogenate, an MIBP (**1**) containing matrix; a typical standard addition calibration for this cultivar is included in Table 1.

2.3. Limit of quantitation (LOQ) and detection (LOD)

The LOQ and LOD were calculated using the amount by which the analyte peak height measured above the baseline ($X_D - X_B$) exceeded the baseline variability (σ_B) $X_D - X_B = K_D \times \sigma_B$. The signal to noise ratio, measured as the division of the corrected signal (height/average noise) and root mean square (RMS) noise, yielded ~3 for a concentration of 0.1 ng L^{-1} MIBP (**1**) in the model juice (Brix 17, pH 3.9), corresponding to the LOD. The LOQ was 2 ng L^{-1} in the model juice because the corresponding qualifier ion m/z = 94 was not visible below this concentration. The juice from Chardonnay, which did not contain any background levels of MIBP (**1**), had the same LOD and LOQ. These LOQ and LOD values would correspond to concentrations of 2.6 pg g^{-1} fresh fruit and 0.13 pg g^{-1} fresh fruit, respectively, after correcting for the dilution of the grapes during homogenization.

Table 1
Calibration curves for non-MIBP containing post-véraison grapes and model juice and typical spiked addition to MIBP-containing Cabernet Sauvignon homogenate.

Sample	Linear equation (0–50 ng L^{-1})	R^2 (R)
Chardonnay juice, 2005 harvest	$Y = 0.0155x + 0.142$	0.99 (0.99)
Pinot noir grape homogenate, 2005 harvest	$Y = 0.0176x - 0.008$	0.99 (0.99)
Model juice, post-véraison; 17 Brix, pH 4.0	$Y = 0.0178x + 0.002$	0.96 (0.98)
Typical Cabernet Sauvignon homogenate, 2005 harvest	$Y = 0.0178x + 0.228$	0.99 (0.98)

2.4. Precision and accuracy

Relative standard deviations (RSD) between three different samples of one batch (120 g) of Cabernet Sauvignon berries were less than 5% (Table 2). Replicate samples from the same homogenate generally had less than 2% variability (Table 2). Spiked recoveries averaged between 91% and 112% (Table 3) and were similar for model juice and all grape samples.

2.5. Matrix influences on pre-véraison, véraison, and post-véraison samples

The juice matrix of Cabernet Sauvignon fruit influenced the measured MIBP (**1**) concentrations (Table 4). The average slopes of the standard addition calibrations in Cabernet Sauvignon berry homogenates were similar regardless of the harvest time; however, the variability in the measured slope response was significantly higher in pre-véraison and véraison samples compared to the post-véraison samples. Average slope for the post-harvest samples was nearly identical to that of a model juice with similar pH and Brix levels (Table 4).

2.6. Detection and quantification of MIBP in 29 grape cultivars during the season

Cabernet Sauvignon, Cabernet franc, Merlot, Sauvignon blanc and Semillon were the only cultivars of the 29 analyzed in which MIBP (**1**) could be quantified (Table 5). All of the cultivars with MIBP (**1**) in the fruit at harvest had higher concentrations in the fruit at the pre-véraison and véraison stages. The concentrations of MIBP (**1**) measured at pre-véraison across cultivars grown in Davis were somewhat less than that (249 ± 28 pg g^{-1} fresh fruit) measured on Cabernet Sauvignon pre-véraison in a commercial vineyard in Napa Valley. There were detectable levels of the MIBP (**1**) quantification ion (m/z = 124) in several other cultivars but there was no evidence of the MIBP (**1**) qualification ion (m/z = 94) in these samples at any time point so that positive identification was not possible.