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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.

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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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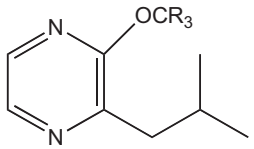

2-methoxy-3-isobutylpyrazine, R = H, (1)
2-($^2D_3$)-methoxy-3-isobutylpyrazine, R = ^{2}D (deuterium), (1a)

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.

Table 2
Determination of method precision. Subsamples A, B and C came from a single sample (120 g) of Cabernet Sauvignon berries. The supernatant from each subsample was split into three replicates.

Subsample	Replications	Peak area		Ratio	Variation by sample in different GC–MS runs			Overall variation for different replications		
		m/z 124	*m/z* 127		Mean	SD*	RSD*	Mean	SD*	RSD*
	1	182,416	437,411	0.417						
A	2	154,315	375,934	0.410	0.410	0.007	2%			
	3	152,848	379,455	0.403						
	1	117,179	257,226	0.456						
B	2	105,984	237,958	0.445	0.450	0.005	1%	0.422	0.022	5%
	3	71,388	159,183	0.448						
	1	73,738	186,467	0.395						
C	2	106,135	258,522	0.411	0.405	0.009	2%			
	3	92,448	225,583	0.410						

* SD and RSD denote standard deviation and relative standard deviation, respectively.

Table 3
Determination of method accuracy in model juice, Cabernet Sauvignon and Pinot noir berry homogenates and Chardonnay juice, $n = 3$. A, B and C are different samples of Cabernet Sauvignon berries.

Samples		Initial MIBP (ng L^{-1})	Spiked (ng L^{-1})	Mean MIBP (ng L^{-1})	% Recovery	Range (ng L^{-1})	SD*	RSD (%)*
Model juice		0	5.0	5.5	109	(5.1–5.8)	0.4	7
Brix 17		0	10.0	9.9	99	(9.7–10.1)	0.3	3
pH 3.9		0	30.0	30.1	100	(29.8–30.5)	0.4	1
		0	50.0	49.9	100	(48.3–51.6)	2.3	5
Cabernet Sauvignon	A	5.1	13.0	18.5	102	(18.4–18.5)	0.0	0
Homogenate	A	5.1	43.5	48.5	100	(48.0–49.1)	0.7	2
Brix 17	B	6.3	13.0	19.6	102	(19.2–20.1)	0.7	3
pH 4	B	6.3	43.5	50.0	101	(49.9–50.1)	0.2	0
	C	12.9	13.0	26.1	101	(25.8–26.5)	0.5	2
	C	12.9	43.5	55.5	98	(54.7–56.3)	1.1	2
Pinot noir		0	4.3	4.7	109	(4.7–4.8)	0.3	1
Homogenate		0	8.7	8.5	98	(8.0–9.1)	0.8	9
Brix 16		0	26.1	25.8	99	(24.8–26.8)	1.4	6
pH 3.7		0	43.5	44.1	101	(43.4–44.8)	1.0	2
Chardonnay juice		0	1.0	1.1	109	(1.1–1.1)	0.5	5
Brix 20.2		0	2.0	2.1	105	(2.0–2.2)	0.1	5
pH 2.98		0	5.0	4.5	91	(4.3–4.6)	0.2	4
		0	10.0	9.8	98	(9.6–10.3)	0.3	4
		0	30.0	33.6	112	(31.3–31.8)	3.6	11
		0	50.0	47.8	95	(49.5–51.6)	5.0	10

* SD and RSD denote standard deviation and relative standard deviation, respectively.

Table 4
Slopes (response ratios) for MIBP (**1**) standard curves prepared in Cabernet Sauvignon grapes at three phenological stages: pre-véraison, véraison and post-véraison and the model juice.

	Cabernet Sauvignon homogenate			Model juice		
	Pre-véraison	Véraison	Post-véraison	Pre-véraison	Véraison	Post-véraison
Mean slope	0.0180	0.0184	0.0176	0.0192	0.0196	0.0178
SD*	0.0103	0.0086	0.0018	0.0008	0.0008	0.0008
RSD*	57%	47%	11%	4%	4%	4%
Brix	3.3	10.2	17.0	3.3	10.2	17.0
pH	2.8	3.2	4.0	2.9	3.0	4.0
Harvest date	7/9/2005	7/30/2005	9/20/2005			
n	30	19	36	3	3	3

* SD and RSD denote standard deviation and relative standard deviation, respectively.

2.7. Approach grafting study

The study was initiated in 2005 primarily to determine the feasibility of approach grafting clusters from field-grown vines onto potted vines. Fruit from three of the treatments reached a soluble solids greater than 21.8 Brix while fruit from the non-grafted, Muscat blanc was only 17.3 Brix (Supplementary Table 1). MIBP (**1**) was detected in the Cabernet Sauvignon fruit of both treatments while MIBP (**1**) for the Muscat blanc fruit was less than the limit of detection.

Methoxypyrazines were found in the berries of Cabernet Sauvignon clusters grafted onto Muscat blanc vines in both 2006 and 2007 (Table 6). The concentrations of MIBP (**1**) in berries of Cabernet Sauvignon clusters grafted onto Muscat blanc vines were 55% the values of Cabernet Sauvignon clusters grafted onto Cabernet Sauvignon vines in 2007. The mean MIBP (**1**) value (12.5 pg g^{-1} fresh weight) of Cabernet Sauvignon clusters grafted onto Muscat blanc vines in 2006 was also 55% the value (23.2 pg g^{-1} fresh weight) of the single replicate Cabernet Sauvignon cluster grafted onto a Cabernet Sauvignon vine that year. While soluble solids

Table 5
Concentrations of MIBP (**1**) in 29 wine-grape cultivars from pre-véraison and véraison and at harvest in 2007. MIBP mean ± standard deviation ($n = 3$).

Cultivars	Pre-véraison		Véraison		Harvest	
	Brix	MIBP[*,**] (pg g^{-1})	Brix	MIBP[*,**] (pg^{-1})	Brix	MIBP[*,**] (pg^{-1})
Alicante	5.2	∠LOD	5.4	∠LOD	26.4	∠LOD
Barbera	5	∠LOD	10.9	∠LOD	28.7	∠LOD
Cabernet Franc	5	184 ± 1.4	12	90 ± 20.6	25.2	9 ± 0.1
Cabernet Sauvignon	4.8	157 ± 23.2	15.1	47 ± 1.7	24.9	13 ± 4.1
Chardonnay	4.7	∠LOD	15.7	∠LOD	24.9	∠LOD
Chenin blanc	3.7	∠LOQ	18	∠LOQ	25.9	∠LOQ
Dolcetto	6.1	∠LOQ	12.7	∠LOD	30.4	∠LOD
Gamay noir	6.3	∠LOD	12.5	∠LOD	28.1	∠LOD
Gewürztraminer	5.7	∠LOQ	10.3	∠LOQ	24.7	∠LOQ
Grenache noir	4.2	∠LOD	12.6	∠LOD	29.3	∠LOD
Malbec	4.1	∠LOQ	14.7	∠LOQ	19.3	∠LOQ
Marsanne	6.4	∠LOD	14.9	∠LOD	27.9	∠LOD
Merlot	6	211 ± 21.9	16.8	38 ± 0.1	25.8	∠LOD
Mourvedre	4	∠LOQ	8.7	∠LOD	22.2	∠LOD
Muscat Alexandria	4.5	∠LOD	5.3	∠LOD	24.3	∠LOD
Muscat blanc	8	∠LOD	8.8	∠LOD	22.9	∠LOD
Nebbiolo	4.8	∠LOQ	6.9	∠LOQ	25.7	∠LOD
Pinot gris	3.7	∠LOD	6	∠LOD	29.1	∠LOD
Riesling	6	∠LOD	13.9	∠LOD	23.9	∠LOD
Roussanne	5.5	∠LOD	5.7	∠LOD	27.3	∠LOD
Sangiovese	6.7	∠LOD	13.5	∠LOD	29	∠LOD
Sauvignon blanc	3.7	94 ± 0.2	13.8	6 ± 1.2	24.3	3 ± 0.1
Semillon	4.7	104 ± 2.7	14.4	5 ± 0.0	28.5	2 ± 0.0
Syrah	4.7	∠LOD	9.8	∠LOD	25.5	∠LOD
Tempranillo	4.4	∠LOD	11.7	∠LOD	26	∠LOD
Torrontes	5.2	∠LOD	10.8	∠LOD	24.9	∠LOD
Verdehlo	3.9	∠LOD	15.6	∠LOD	29	∠LOD
Viognier	4.3	∠LOD	19.1	∠LOD	27.6	∠LOD
Zinfandel	4.5	∠LOD	12	∠LOD	27.7	∠LOD

All concentrations determined using model juice matrices with corresponding pH and Brix levels for each harvest.
[*] ∠LOD = below limit of detection.
[**] ∠LOQ = below limit of quantification.

were similar across the Cabernet graft configuration treatments in 2006 and 2007, berries from non-grafted clusters had the lowest MIBP values both years.

There were differences in soluble solids of Muscat blanc fruit at harvest in 2005 and 2007 between the two grafting treatments. The non-grafted Muscat blanc fruit was harvested at 17 Brix compared to 23.5 Brix for the grafted Muscat clusters on Cabernet Sauvignon in 2005 (Table 6, Supplementary Table 1). The opposite occurred in 2007 where Muscat blanc clusters grafted onto Cabernet Sauvignon vines did not ripen properly and were harvested at ~16 Brix compared to the non-grafted fruit which was harvested at ~21.5 Brix (Table 6).

3. Discussion

In this study, a method for extracting and measuring MIBP (**1**) concentration in whole berries throughout maturation was developed that includes use of standard additions to eliminate unacceptable noise in the MIBP (**1**) response curves in unripe fruit. The method was used to evaluate 29 cultivars for the presence of MIBP (**1**) in unripe and ripe fruit, and to show that the fruit genotype, and not the shoot genotype, determines the presence or absence of MIBP (**1**) in ripe fruit.

3.1. Grape sample preparation

In this study, MIBP (**1**) levels were measured in the whole grape berry. Seeds can contain high levels of MIBP (**1**) (Roujou de Boubée et al., 2002), so separation of seeds from the grape berry prior to analysis, or analysis of skins only may be appropriate for some experimental objectives. Use of frozen berries, without thawing prior to homogenization, provided for a rapid sample preparation time of ~10 min from weighing to the end of centrifugation.

Berries were homogenized using a tissue homogenizer with a Saw Tooth Probe. Other tissue homogenization procedures were evaluated, including a small potato ricer and a household coffee grinder. The ricer was not able to release measurable levels of MIBP (**1**) from the skins and the blender and grinder were not as effective as the homogenizer in breaking down berry tissue. A tissue sonicator was also tested but it was unable to break up the larger berries. Improved recoveries may be obtained with the Omni Homogenizer used in this study by increasing the homogenization time to create smaller particle sizes or by addition of ethanol or other solvents to the berries prior to homogenization, however, these variables were not evaluated in this study.

The deuterated MIBP (**1a**) internal standard was added to the berries prior to homogenization to provide a quantitative control throughout the extraction procedure. This differs from more recent methodologies that added the internal standard later in the process (Belancic and Agosin, 2007; Romero et al., 2006; Ryona et al., 2008, 2009; Sala et al., 2000). Analysis of samples spiked with known MIBP (**1**) concentrations resulted in recoveries of 91–112%, indicating the method is highly accurate for a range of matrices including a model juice and homogenates or juices from post-harvest MIBP-containing and non-MIBP-containing grape cultivars. This recovery is similar to that reported by Belancic and Agosin (2007) although their SPME extraction time was longer (1 h), and greater than the 86% recovery reported by Hashizume and Umeda (1996) using a distillation and solvent extraction procedure. The sample preparation procedure was also highly reproducible with relative standard deviations of 5% for replicate grape samples and <2% for analysis of multiple aliquots of the supernatant from the same initial grape sample.

Table 6
Soluble solids and the concentrations of MIBP (**1**) measured in fruit from the approach grafting study in 2006 and 2007. Clusters of Cabernet Sauvignon were grafted onto Muscat blanc vines and Cabernet Sauvignon vines. Muscat blanc clusters were grafted onto Cabernet Sauvignon and Muscat blanc vines. Also included are values obtained from non-grafted clusters. Clusters were grafted from a field-grown vine onto a potted host vine except where indicated by a * in the replicate column. In that situation, a cluster from a potted vine was grafted onto a field-grown vine. The concentration of MIBP (**1**) is expressed on a g fresh berry weight basis.

Graft configuration	2006			2007		
	Replicate	Soluble solids (Brix)	MIBP (**1**) (pg g^{-1})	Replicate	Soluble solids (Brix)	MIBP (**1**) (pg g^{-1})
Cabernet Sauvignon fruit on Muscat blanc	1*	16.3	15.9	1	23.2	12.5
	2	19.8	13.1	2*	22.5	12.4
	3	22.7	12.2	3	24.2	5.6
	4	24.9	8.9	4	23.1	14.4
Mean ± SD		20.9 ± 1.9	12.5 ± 1.4		23.2 ± 0.4	11.2 ± 1.9
Cabernet Sauvignon fruit on Cab. Sauv.	1	19.9	23.2	1	23.3	18.9
				2	22.6	24.3
				3*	23.2	19.6
				4	22.9	20.0
Mean ± SD		19.9	23.2		23.0 ± 0.2	20.7 ± 1.2
Cabernet Sauvignon non-grafted fruit	1	22.4	7.1	1	22.8	5.4
	2	22.4	7.6	2	22.7	5.0
	3	22.9	11.1	3	23.3	6.9
	4	22.8	13.5	4	23.0	7.0
Mean ± SD		22.6 ± 0.1	9.8 ± 1.5		23.0 ± 0.1	6.1 ± 0.5
Muscat blanc fruit on Cabernet Sauvignon	1	28.9	∠LOD[a]	1	14.2	∠LOD[a]
	2	23.8	∠LOD	2*	14.9	∠LOD
	3	25.2	∠LOD	3	17.0	∠LOD
	4	25.7	∠LOD	4	17.5	∠LOD
	5	22.8	∠LOD			
	6*	23.6	∠LOD			
Mean ± SD		25.0 ± 0.9	∠LOD		15.9 ± 0.8	∠LOD
Muscat blanc fruit on Muscat blanc				1	24.1	∠LOD
				2	17.2	∠LOD
Mean ± SD					20.7	∠LOD
Muscat blanc non-grafted fruit	1	22.2	∠LOD	1	22.9	∠LOD
	2	22.8	∠LOD	2	17.3	∠LOD
	3	22.0	∠LOD	3	23.9	∠LOD
	4	21.7	∠LOD	4	21.4	∠LOD
Mean ± SD		21.2 ± 0.2	∠LOD		21.4 ± 1.4	∠LOD

[a] MIBP concentrations were below the limit of detection (LOD).

3.2. Influence of grape matrix on quantitation

Based on variability in response ratios observed in pre-véraison and véraison samples, errors of 140% or more in measured MIBP (**1**) levels are possible if effects of matrix pH and Brix levels are not considered. Here, we used the method of standard addition to minimize the effects of this matrix variability and to quantify MIBP (**1**) in the fruit. All of the initial method validation studies were done using grapes harvested post-véraison or in model juices that simulated the Brix and pH of a post-véraison grape juice. During analysis of grape samples harvested pre-véraison and at véraison, we observed significant variability in the response ratios (slopes) of standard curves prepared using grapes harvested at different maturity levels. This slope variability was not observed in post-véraison samples or in model juice samples that simulated the Brix and pH at the various harvest times. The reason for this variability in response for pre-véraison grape homogenates is not clear, however it may be due to the low (<3.8) and variable pH of fruit prior to véraison. Hartmann et al. (2002) observed that HS-SPME extraction of MIBP (**1**) from model wines decreased significantly below pH 3 and increased significantly above pH 11. Ryona et al. (2009) adjusted the sample pH with 20% NaOH prior to HS-SPME analysis and observed no significant matrix effects on MIBP (**1**) analysis in pre-véraison berries compared to ripe berries at harvest; in their study, adjustments of juice pH from pH 2 to 9 did not significantly impact recovery. Further analysis of the effect of pH on extraction of MIBP (**1**) from grape samples by HS-SPME is needed to clarify the pH effects in pre-véraison fruit matrices. Use of a single calibration curve may be appropriate for post-harvest samples as demonstrated by the similarity in responses for a model juice (pH 4 and 17 Brix) and post-harvest juice samples (Table 5). However, to minimize the effects of this matrix variability on quantitation of MIBP, the standard addition method of calibration and quantitation is recommended (Skoog et al., 1992).

3.3. Analysis of MIBP (*1*) in 29 *V. vinifera* cultivars

In a survey of 29 grape cultivars grown in the experimental vineyards in Davis, CA, MIBP (**1**) was present in quantifiable amounts only in Cabernet franc, Cabernet Sauvignon, Merlot, Sauvignon blanc and Semillon. In addition a peak corresponding to the MIBP (**1**) quantification ion (m/z = 124) was identified in Chenin blanc, Gewürztraminer, and Malbec, but detection of MIBP (**1**) in these cultivars cannot be confirmed because the qualification ion (m/z = 94) was not present. Further study of these cultivars is needed. To our knowledge this is the most comprehensive study of MIBP (**1**) in grape cultivars to date. It is important to note that in the current study all rachises and pedicels were carefully removed prior to homogenization and HS-SPME-GC–MS-SIM analysis. Rachises and pedicels may be an important source of MIBP (**1**) in Cabernet Sauvignon (Roujou de Boubée et al., 2002) and Chardonnay wines (Hashizume and Samuta, 1997).

The pre-véraison MIBP (**1**) concentrations and those determined at harvest in this study are similar to those reported by others in Cabernet Sauvignon and Merlot from Bordeaux, France, and Tarragona, Spain; Sauvignon blanc from Wagga Wagga, Australia; and Cabernet franc from New York, USA (Allen and Lacey, 1993; Kotseridis et al., 1999; Lacey et al., 1991; Roujou de Boubée et al., 2000; Ryona et al., 2008; Sala et al., 2000). In this study, the MIBP concentration in cultivars with detectable MIBP (**1**)

decreased from just before véraison until harvest. Similar patterns during maturation have been reported by others (Allen and Lacey, 1993; Hashizume and Samuta, 1999; Lacey et al., 1991; Ryona et al., 2008).

Of the 29 cultivars, 10 have been tested previously for MIBP in fruit, including eight in one study by Hashizume and Samuta (1999) but questions regarding the presence or absence of pedicels and other methodological issues impair firm conclusions. Hashizume and Samuta (1999) reported MIBP in Cabernet Sauvignon, Merlot, Sauvignon blanc, and Semillon, similar to the present study. However, in that study, MIBP was also present in unripe berries of Chardonnay and Riesling, in contrast to our analysis. Hashizume and Samuta (1999) analyzed grape tissues (juice extracted from crushed berries and combined skin and pulp samples) using a distillation and extraction procedure followed by GC–MS-SIM. The method has a reported MIBP detection limit of <0.2 ng kg^{-1} tissue and a recovery of 86% from grape juice; variability in response was not reported for juice samples but was <2% for red wine (Hashizume and Umeda, 1996). The authors did not report method performance parameters for berries obtained prior to harvest maturity, however; 2-methyl-3-*n*-propylpyrazine was used as an internal standard rather than a stable isotope labeled MIBP analog, although Allen et al. (1994) have indicated that methoxypyrazines with different alkyl side chains may not act reliably as internal standards for endogenous methoxypyrazines. In addition, Hashizume and Samuta (1999) did not clarify their use of the ions *m*/*z* = 94 and 124 for quantification and qualification of MIBP (**1**) chromatographic peaks therefore their identification of low levels of MIBP (**1**) in these cultivars may not be confirmed. Although Allen and Lacey (1993) also report the presence of MIBP (**1**) in juice obtained from crushed Chardonnay and Riesling berries, no actual concentrations were reported for these cultivars. The method used by Allen and Lacey has been the most widely used procedure prior to development of SPME methodology. In this method, juice from crushed grapes is distilled and extracted and a stable isotope internal standard combined with chemical ionization MS is used to provide an MIBP detection limit of <0.2 ng L^{-1}. Again, however, the method validation parameters have not been reported for unripe grapes. Furthermore, the presence or absence of stems during crushing was not specified (Allen and Lacey, 1993). More work characterizing MIBP (**1**) levels in stems for these and other grape cultivars is needed in order to determine the potential for its extraction from stems during fermentation.

Carmenere was reported to contain MIBP (**1**) with high concentrations of about 100 ng kg^{-1} berry fresh weight (Belancic and Agosin, 2007), and recently Ryona et al. (2008) added Cabernet franc. Thus, Cabernet Sauvignon, Cabernet franc, Merlot, Sauvignon blanc, Semillon, and Carmenere are cultivars with measurable MIBP (**1**) in mature berries, and there may be small amounts in unripe berries of Chardonnay and Riesling. This phenotypic information combined with genetic information such as that obtained from determining grapevine parentage (Bowers and Meredith, 1997) and new information from the fully sequenced Pinot noir genome (Jaillon et al., 2007; Velasco et al., 2007) may provide the basis for a more complete understanding of the genetic basis for the occurrence of MIBP (**1**) in grapes. In that regard, it should be noted that Cabernet franc, Cabernet Sauvignon, and Sauvignon blanc are closely related (Bowers and Meredith, 1997); Cabernet franc, Merlot, and Carmenere are related (Boursiquot et al., 2009).

3.4. Approach grafting study

This study demonstrates that the biosynthesis of MIBP (**1**) in Cabernet Sauvignon berries and most likely in the other MIBP-producing cultivars (Cabernet franc, Carmenere, Merlot, Semillon, and Sauvignon blanc) is independent of the genotype of other vine organs and is therefore determined by genotype of the fruit. In reciprocal grafting experiments, MIBP (**1**) was found in all Cabernet Sauvignon clusters in all 3 years, regardless of whether the cluster developed *in situ*, grafted onto a Cabernet Sauvignon shoot, or grafted onto a Muscat blanc shoot. Correspondingly, MIBP (**1**) was never found in Muscat blanc clusters regardless of the genotype of the shoot on which they developed. Thus, although MIBP (**1**) is produced in grapevine leaves, it was not translocated to the (Muscat blanc) fruit. Grafting was completed early enough to allow significant translocation and accumulation, and fruit developed normally. This study was designed as shown by Gholami et al. (1995), and the results show that MIBP (**1**) is similar to monoterpenes in that they are also synthesized in the leaves (Gunata et al., 1986), but are not translocated to fruit (Gholami et al., 1995). Together these grafting experiments show that, to the extent we understand berry ripening, flavor compounds are synthesized in the berries and are not translocated to the fruit from leaves or soil.

4. Concluding remarks

We developed a method for analysis of MIBP (**1**) in whole grape berries that is accurate (spiked recoveries of >91%), reproducible (RSD <10%), and sensitive (LOQ = 2 ng L^{-1}). However, the grape matrix, particularly for pre-véraison and véraison samples, can influence the accuracy and precision of the analysis, therefore, the method of standard additions is recommended for quantification. Reciprocal grafting experiments demonstrated that MIBP (**1**) is produced in the berries of Cabernet Sauvignon and is not produced in the berries of Muscat blanc irrespective of the genotype of the shoot upon which the fruit develop. Thus, the fruit genotype is responsible for the presence of MIBP in the fruit, implying that the synthesis occurs in the fruit and that MIBP is not translocated to the fruit from the shoot.

5. Experimental

5.1. General experimental procedures

MIBP (99% pure; Fig. 1) for preparation of standards was purchased from Sigma Chemical Co. (St. Louis, MO). The internal standard 2-($^2D_3$)-methoxy-3-isobutylpyrazine (dMIBP**1a**; Fig. 1) was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada, 98% atom% D). SPME fibers (23 gauge, 2 cm divinylbenzene/Carboxen™/polydimethylsiloxane (DVB/CARB/PDMS)) were purchased from Supelco, Bellafonte, PA. An Agilent 6890 GC with a 5973MSD (Agilent, Santa Clara, CA) and Gerstel MPS2 autosampler (Gerstel Inc., Columbia, MD) were used for all analyses. GC–MS conditions are provided in Section 5.5.

5.2. Fruit

Fruit from a *V. vinifera* L. cv. Cabernet Sauvignon (clone 7) commercial vineyard near Rutherford, CA was used for method validation; grapes (3 kg) were harvested at commercial maturity (24 Brix) in 2005. The effect of fruit maturation on the analysis of MIBP (**1**) was determined by harvesting clusters from the south facing side of the canopy in the same vineyard pre-véraison, véraison and at harvest. For comparison of MIBP concentrations in different cultivars, fruits from 29 *V. vinifera* cultivars were harvested from the demonstration vineyard at the University of California, Davis; for pre-véraison (July 2, 2007), véraison (July 25, 2007) and harvest (August 28 and September 3, 2007) samples, four whole clusters from the south facing side of the canopy of each cultivar were harvested at random from a row of 35 vines. For all experiments,

clusters were placed into a cooler with dry ice and taken to the laboratory. Berries from the clusters were removed from the stems, mixed, and randomly transferred into 50 mL Falcon™ tubes (Becton, Dickinson and Co., NJ) or plastic bags. Prior to freezing, soluble solids were measured on a sub-sample of berries with a Reichert AR200 refractometer (Reichert Analytical Instruments, Depew, NY). Berries were stored at −80 °C until MIBP analysis.

Unsulfited Chardonnay juice (20.1 Brix, pH 2.98) was obtained from the UC Davis experimental winery from grapes grown at the UC Davis vineyards. This juice was used as a non-MIBP containing matrix for method validation and calibration studies.

5.3. Grape sample preparation

Berries (36 g) were used for each sample. The actual number of berries in the sample varied from 30 to 120, depending upon developmental stage and cultivar. The frozen, whole berries (36 g) were weighed and placed in a 50 mL plastic centrifuge tube. An aliquot of a 2 mM NaF solution (10.0 mL) prepared using MilliQ™ filtered water (Millipore, Bedford, MA) and containing 200 ng L^{-1} of internal standard, 2-($^2D_3$)-methoxy-3-isobutylpyrazine **1a** (dMIBP; Fig. 1), was added to the berries in the tube. The solution was kept on ice and homogenized until smooth—approximately 40 s at a speed of 3.5 on the manufacturer's scale with an Omni Homogenizer GLH 80 equipped with a 20 mm × 195 mm Saw Tooth (Fine) Generator Probe Model #G20-195ST (Omni International, Marietta, GA). The temperature of the solution after homogenizing was ~2 °C. The homogenate was then centrifuged (Eppendorf Model 5403, Westbury, NY, USA) at 4137 × g for 5 min at 4 °C. At least five replicates of 36 g each were prepared for each analysis.

Following centrifugation, aliquots (10.0 mL) of the supernatant were transferred to each of three separate 20 mL glass, round-bottom, amber, screw cap headspace vial (Supelco, Bellefonte, PA) containing 3.0 g NaCl. The vial was then closed tightly and allowed to equilibrate for at least 1 h at 20 °C before HS-SPME-GC–MS analysis. Five replicates of berries (36 g) were prepared.

5.4. Model juice preparation

Model juices were prepared by adding D (−) fructose (Sigma Aldrich, St. Louis, MO) and L (+) tartaric acid (Sigma–Aldrich, St. Louis, MO) to MilliQ™ purified water until the desired soluble solids and pH were obtained. The model juices were prepared to simulate the Brix and pH of juice from pre-véraison (3.3 Brix, pH 2.9), véraison (10.2 Brix, pH 3.02), and post-véraison (17 Brix, pH 4.0) grape samples.

5.5. HS-SPME-GC–MS analysis

The basic conditions of Chapman et al. (2004) were used for all analyses. Extractions were performed using a 23 gauge, 2 cm divinylbenzene/Carboxen™/polydimethylsiloxane (DVB/CARB/PDMS) SPME fiber, that was conditioned and cleaned according to manufacturer's specifications. The SPME fiber was exposed to the headspace of each sample vial and the sample extracted for 30 min at 40 °C with continuous agitation. The SPME fiber was then removed from the vial and placed into the GC–MS inlet equipped with a 0.7 mm straight glass liner. The inlet was held at 260 °C in splitless mode for 5 min for the analytes to desorb from the fiber. Then the inlet flow was switched on at 50 mL min^{-1} with the fiber in the inlet for an additional 5 min (no carry over was detected).

An HP 5MS capillary column (30 m length × 0.25 mm ID; 0.25 μm film thickness; Agilent) was used for separation. The oven temperature was maintained at a constant temperature of 40 °C for 5 min, then increased 2.5 °C min^{-1} to 80 °C, 5 °C min^{-1} to 110 °C, and 25 °C min^{-1} to 230 °C before holding steady for 5 min. The MSD interface was held at 280 °C and the carrier gas was He at a constant pressure of 4.77 psi with a nominal initial flow of 0.8 mL min^{-1} and average linear velocity of 32 cm s^{-1}. Selected ion monitoring (SIM) was used at mass channels of m/z = 94 and 124 for MIBP (**1**) and m/z = 127 and 154 for dMIBP (**1a**). Peak areas of the ions m/z 124 and 127 were used for quantification and ions m/z 94 and 154 were used for qualification. Retention times for dMIBP (**1a**) and MIBP (**1**) were ~26.17 min and ~26.23 min, respectively.

5.6. External standard calibration and quantification by standard addition

Standard MIBP (**1**) calibration samples were prepared in model juice, Chardonnay juice, or Pinot noir homogenate to give concentrations of 0, 0.1, 0.5, 1, 2, 5, 15, 30, and 50 ng L^{-1}. The internal standard (IS), dMIBP (**1a**), was also added to each standard at a concentration of 50 ng L^{-1}. A 10.0 mL aliquot of each standard was transferred into a 20 mL headspace vial that contained NaCl (3 g). At least three aliquots of each standard were analyzed by HS-SPME-GC–MS as described above. Peak area ratios of MIBP (**1**) and dMIBP (**1a**) were used to create linear calibration curves.

MIBP (**1**) concentrations in grape homogenates were determined by the method of standard addition. Frozen, whole berries were prepared as described above, except the aliquot of the 2 mM NaF/200 ng L^{-1} dMIBP (**1a**) (10.0 mL) solution also contained MIBP (**1**) at a concentration of either 0, 4.0, 8.0, 20, 40, 60, 120 or 200 ng L^{-1}. The supernatant was analyzed by HS-SPME-GC–MS as described above. The peak area ratio of MIBP (**1**) relative to dMIBP (**1a**) was used to create a standard addition calibration curve for each sample after correcting the standard concentration for the dilution by the grapes. The concentration of MIBP (**1**) in the supernatant was calculated from the linear regression equation of each calibration curve at the point where the y-intercept is equal to zero. MIBP (**1**) concentrations originally in the fruit were calculated from the supernatant concentration by correcting for the dilution of the original 36 g of grape sample with 10.0 mL of aqueous dMIBP/MIBP (**1a**/**1**) solution and assuming a density of 1.0 g mL^{-1} for the standard solution. Corrected concentrations in grape were expressed as pg MIBP (**1**) per g fresh fruit.

5.7. Precision and accuracy

To determine the reproducibility of the sample preparation procedure, a sample of frozen Cabernet Sauvignon berries (120 g) was divided into three different subsamples of 36 g each. The grapes were placed into plastic centrifuge tubes, homogenized with the dMIBP (**1a**) solution (no MIBP (**1**) was added) and centrifuged as described previously. The supernatant from each subsample was split into three 10.0 mL aliquots, each of which were added to 20 mL amber glass headspace vials containing NaCl (3 g). The vials were analyzed by HS-SPME-GC–MS analysis as described above. The absolute peak areas and peak area ratios of MIBP (**1**) and dMIBP (**1a**) for the replicates were averaged and precision was reported as the standard deviation and relative standard deviation.

Accuracy of the method was determined in model juice, Chardonnay juice, Cabernet Sauvignon homogenate and Pinot noir homogenate by spiking known concentrations of MIBP into the juice or homogenate, correcting for the un-spiked "zero" value, and relating the measured MIBP to the known spike amount.

5.8. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantification (LOQ) for MIBP (**1**) were reported as the concentration where the calculated signal to noise ratio of the quantifier ion (m/z = 124)

was at least 3 and 10, respectively. The LOQ also required the presence of the qualifier ion m/z = 94. Values are reported in ng L^{-1} corresponding to the concentration in the juice or model wine.

5.9. Approach grafting study

Potted and field-grown *V. vinifera* L. cvs. Cabernet Sauvignon and Muscat blanc (syn. Moscato bianco, Muscat de Frontignan) grapevines were used in the study which was conducted in 2005, 2006 and 2007. The potted vines were secured below the cordons of Cabernet Sauvignon and Muscat blanc vines trained to a vertical shoot positioning (VSP) trellis system at the Department of Viticulture and Enology's experimental vineyard on the University of California at Davis campus. Cabernet Sauvignon and Muscat blanc clusters from a donor vine were approach grafted to Muscat blanc and Cabernet Sauvignon host vines.

Clusters were approach grafted prior to anthesis. The majority of the individual replicates were the result of grafting clusters from field-grown vines onto potted vines. In a few instances clusters from potted vines were grafted onto field-grown vines. Special care was taken to insure good cambium contact to facilitate callus formation and to avoid desiccation of the graft union. Two to three weeks after grafting (when the graft union had callused over and berry set had taken place), the donor cane was severed, leaving the donor cluster and a piece of cane attached to the host vine. The potted vines with the donor clusters remained attached to the donor vines' cordons. Therefore the potted vines' shoots grew within the donor vines' canopy in the field. The pots were watered almost daily during the remainder of the growing season. The donor cluster developed on the host vine until the fruit reached maturity.

Clusters were harvested at approximately 22 Brix and put in a container with dry ice and transported to the Department of Viticulture and Enology and stored at −65 °C until analyses were performed. Berries (36 g) were used for MIBP (**1**) analysis. MIBP (**1**) was analyzed as described above. Soluble solids were determined on berry samples with a temperature compensating refractometer (Reichert model AR200 digital refractometer, Reichert Analytical Instruments, Depew, NY).

5.10. Statistical analysis

Data were analyzed via ANOVA and means were compared using Fisher's LSD (SAS, Cary, NC). Differences were considered significant at the 95% level. Linear regressions were performed with Sigma Plot (SPSS Inc., Chicago, IL) or SAS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.09.006.

References

Allen, M.S., Lacey, M.J., 1993. Methoxypyrazine grape flavour: influence of climate, cultivar and viticulture. Die Wein-Wissenschaft 48, 211–213.

Allen, M.S., Lacey, M.J., 1999. Methoxypyrazines of grapes and wines. In: Waterhouse, A.L., Ebeler, S.E. (Eds.), Chemistry of Wine Flavor. American Chemical Society, Washington, DC, pp. 31–38.

Allen, M.S., Lacey, M.J., Boyd, S., 1994. Determination of methoxypyrazines in red wines by stable-isotope dilution gas-chromatography mass-spectrometry. J. Agric. Food Chem. 42, 1734–1738.

Augustyn, O.P.H., Rapp, A., Van Wyk, C.J., 1982. Some volatile aroma components of *Vitis vinifera* L. cv. Sauvignon blanc. S. Afr. J. Enol. Vitic. 3, 53–60.

Bayonove, C., Cordonnier, R., Dubois, P., 1975. Study of an aromatic characteristic fraction of Cabernet Sauvignon grape variety, identification of 2-methoxy-3-isobutylpyrazine. Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sci. Serie D 281, 75–78.

Belancic, A., Agosin, E., 2007. Methoxypyrazines in grapes and wines of *Vitis vinifera* cv. Carmenere. Am. J. Enol. Vitic. 58, 462–469.

Boursiquot, J.-M., Lacombe, T., Laucou, V., Julliard, S., Perrin, F.-X., Lanier, N., Legrand, D., Meredith, C., This, P., 2009. Parentage of Merlot and related winegrape cultivars of southwestern France: discovery of the missing link. Austr. J. Grape Wine Res. 15, 144–155.

Bowers, J.E., Meredith, C.P., 1997. The parentage of a classic wine grape, Cabernet Sauvignon. Nat. Genet. 16, 84–87.

Buttery, R.G., Seifert, R.M., Guadagni, D.G., Ling, L.C., 1969. Characterization of some volatile constituents of bell peppers. J. Agric. Food Chem. 17, 1322–1327.

Chapman, D.M., Thorngate, J.H., Matthews, M.A., Guinard, J.X., Ebeler, S.E., 2004. Yield effects on 2-methoxy-3-isobutylpyrazine concentration in Cabernet Sauvignon using a solid phase microextraction gas chromatography/mass spectrometry method. J. Agric. Food Chem. 52, 5431–5435.

Ebeler, S.E., 2001. Analytical chemistry: unlocking the secrets of wine flavor. Food Rev. Int. 17, 45–64.

Gholami, M., Hayasaka, Y., Coombe, B.G., Jackson, J.F., Robinson, S.P., Williams, P.J., 1995. Biosynthesis of flavour compounds in Muscat Gordo Blanco grape berries. Austr. J. Grape Wine Res. 1, 19–24.

Gunata, Y.Z., Bayonove, C.L., Baumes, R.L., Cordonnier, R.E., 1986. Changes in free and bound fractions of aromatic components in vine leaves during development of muscat grapes. Phytochemistry 25, 943–946.

Harris, R.L.N., Lacey, M.J., Brown, W.V., Allen, M.S., 1987. Determination of 2-methoxy-3-alkylpyrazines in wine by gas chromatography/mass spectrometry. Vitis 26, 201–207.

Hartmann, P.J., McNair, H.M., Zoecklein, B.W., 2002. Measurement of 3-alkyl-2-methoxypyrazine by headspace solid-phase microextraction in spiked model wines. Am. J. Enol. Vitic. 53, 285–288.

Hashizume, K., Samuta, T., 1997. Green odorants of grape cluster stem and their ability to cause a wine stemmy flavor. J. Agric. Food Chem. 45, 1333–1337.

Hashizume, K., Samuta, T., 1999. Grape maturity and light exposure affect berry methoxypyrazine concentration. Am. J. Enol. Vitic. 50, 194–198.

Hashizume, K., Umeda, N., 1996. Methoxypyrazine content of Japanese red wines. Biosci. Biotechnol. Biochem. 60, 802–805.

Jaillon, O., Aury, J.M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., Choisne, N., Aubourg, S., Vitulo, N., Jubin, C., Vezzi, A., Legeai, F., Hugueney, P., Dasilva, C., Horner, D., Mica, E., Jublot, D., Poulain, J., Bruyere, C., Billault, A., Segurens, B., Gouyvenoux, M., Ugarte, E., Cattonaro, F., Anthouard, V., Vico, V., Del Fabbro, C., Alaux, M., Di Gaspero, G., Dumas, V., Felice, N., Paillard, S., Juman, I., Moroldo, M., Scalabrin, S., Canaguier, A., Le Clainche, I., Malacrida, G., Durand, E., Pesole, G., Lauacou, V., Chatelet, P., Merdinoglu, D., Delledonne, M., Pezzotti, M., Lecharny, A., Scarpelli, C., Artiguenave, F., Pe, M.E., Valle, G., Morgante, M., Caboche, M., Adam-Blondon, A.F., Weissenbach, J., Quetier, F., Wincker, P.French-Italian Public Consortium for Grapevine Genome Characterization, 2007. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449, 463–467.

Kotseridis, Y., Baumes, R.L., Bertrand, A., Skouroumounis, G.K., 1999. Quantitative determination of 2-methoxy-3-isobutylpyrazine in red wines and grapes of Bordeaux using a stable isotope dilution assay. J. Chromatogr., A 841, 229–237.

Lacey, M.J., Allen, M.S., Harris, R.L.N., Brown, W.V., 1991. Methoxypyrazines in Sauvignon blanc grapes and wines. Am. J. Enol. Vitic. 42, 103–108.

Pawliszyn, J., 1997. Solid Phase Microextraction: Theory and Practice. Wiley-VCH Inc., New York, NY.

Romero, R., Chacon, J.L., Garcia, E., Martinez, J., 2006. Pyrazine contents in four red grape varieties cultivated in a warm climate. J. Int. Des Sci. De La Vigne Et Du Vin 40, 203–207.

Roujou de Boubée, D., Van Leeuwen, C., Dubourdieu, D., 2000. Organoleptic impact of 2-methoxy-3-isobutylpyrazine on red Bordeaux and Loire wines. Effect of environmental conditions on concentrations in grapes during ripening. J. Agric. Food Chem. 48, 4830–4834.

Roujou de Boubée, D., Cumsille, A.M., Pons, M., Dubourdieu, D., 2002. Location of 2-methoxy-3-isobutylpyrazine in Cabernet Sauvignon grape bunches and its extractability during vinification. Am. J. Enol. Vitic. 53, 1–5.

Ryan, D., Watkins, P., Smith, J., Allen, M., Marriott, P., 2005. Analysis of methoxypyrazines in wine using headspace solid phase microextraction with isotope dilution and comprehensive two-dimensional gas chromatography. J. Sep. Sci. 28, 1075–1082.

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

Ryona, I., Pan, B.S., Sacks, G.L., 2009. Rapid measurement of 3-alkyl-2-methoxypyrazine content of winegrapes to predict levels in resultant wines. J. Agric. Food Chem. 57, 8250–8257.

Sala, C., Mestres, M., Marti, M.P., Busto, O., Guasch, J., 2000. Headspace solid-phase microextraction method for determining 3-alkyl-2-methoxypyrazines in musts by means of polydimethylsiloxane-divinylbenzene fibres. J. Chromatogr., A 880, 93–99.

Skoog, D.A., Holler, J.F., Nieman, T.A., 1992. Principles of Instrumental Analysis. Saunders College Publishing, Philadelphia, PA.

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D.A., Cestaro, A., Pruss, D., Pindo, M., FitzGerald, L.M., Vezzulli, S., Reid, J., Malacarne, G., Iliev, D., Coppola, G., Wardell, B., Micheletti, D., Macalma, T., Facci, M., Mitchell, J.T., Perazzolli, M., Eldredge, G., Gatto, P., Oyzerski, R., Moretto, M., Gutin, N., Stefanini, M., Chen, Y., Segala, C., Davenport, C., Demattè, L., Mraz, A., Battilana, J., Stormo, K., Costa, F., Tao, Q., Si-Ammour, A., Harkins, T., Lackey, A., Perbost, C., Taillon, B., Stella, A., Solovyev, V., Fawcett, J.A., Sterck, L., Vandepoele, K., Grando, S.M., Toppo, S., Moser, C., Lanchbury, J., Bogden, R., Skolnick, M., Sgaramella, V., Bhatnagar, S.K., Fontana, P., Gutin, A., Van de Peer, Y., Salamini, F., Viola, R., 2007. A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS One 2, e1326.