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Survey of 3-Alkyl-2-methoxypyrazine Content of South African Sauvignon Blanc Wines Using a Novel LC−APCI-MS/MS Method

Philippus Alberts,[†,‡] Maria A. Stander,[§] Sylvia O. Paul,[‖] and
Andre de Villiers*,[†]

[†]Department of Chemistry and Polymer Science, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa, [‡]National Department of Agriculture, Private Bag X5015, Stellenbosch, 7600, South Africa, [§]Central Analytical Facility, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa, and [‖]Department of Chemistry, University of South Africa, P.O. Box 392, UNISA, 0003, South Africa

An LC−MS/MS method for the trace-level determination of 3-alkyl-2-methoxypyrazines in Sauvignon blanc wines is described. 3-Isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (SBMP) were analyzed by reversed phase liquid chromatography coupled to atmospheric pressure chemical ionization, as electrospray ionization was found to suffer from matrix quenching effects. A sample preparation method involving distillation of wine followed by solvent extraction and sufficient preconcentration was developed. Limits of detection and quantification for all three analytes were 0.03 ng/L and 0.10 ng/L, respectively, making the method more sensitive than gas chromatographic methods. IBMP was found to be the most abundant congener in South African Sauvignon blanc wines, with concentrations varying between 0.40 and 44 ng/L in 575 samples. IPMP and SBMP levels varied from <0.03 to 3.9 and <0.03 to 3.2 ng/L, respectively. Statistical investigation indicated no clear correlation between methoxypyrazine content and either geographical origin or vintage. The method was also successfully applied for the quantitation of IBMP in five additional South African wine varieties, including three red wine cultivars. The developed method represents a powerful new tool for the in-depth investigation of these important wine aroma constituents.

KEYWORDS: Methoxypyrazines; wine; Sauvignon blanc; LC−MS/MS; multiple reaction monitoring (MRM)

INTRODUCTION

Pyrazines (1,4-diazines, **Figure 1**) are nitrogen-containing heterocyclic compounds that are widely distributed in nature. Various 3-alkyl-2-methoxypyrazines (MPs) have been identified in materials of vegetable origin, where they contribute significantly to the characteristic aroma of the products (*1, 2*). Three MPs, namely, 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (SBMP), are particularly dominant in natural products (*2*). In many cases all three MPs are present, with one compound often clearly dominant. In green and red peppers as well as wine grapes IBMP predominates, while SBMP is the dominant pyrazine in carrot, parsnip and beetroot and IPMP in peas, broad beans and cucumber (*2*). 3-Ethyl-2-methoxypyrazine (EMP) and 3-methyl-2-methoxypyrazine (MMP) have also been identified in natural products (*1*). The odor threshold levels of the relevant MPs in water vary between 1 to 2 ng/L (IBMP, IPMP and SBMP), 425 ng/L (EMP) and 4000 ng/L (MMP) (*1−3*).

Sauvignon blanc wine is characterized by an aroma described *inter alia* as nuances of green, grassy, herbaceous and green pepper (*3, 4*). MPs are known to contribute to the distinctive aroma associated with wine of this cultivar (*3, 4*). The most abundant congener in Sauvignon blanc wine is IBMP, representing approximately 80% of the total MP content. The other principal MPs commonly found in wine, SBMP and IPMP, each represent approximately 10% of the total MP content (*3, 4*). MPs are important wine flavor components due to their extremely low sensory detection thresholds: 1 to 2 ng/L of IBMP has a significant influence on the aroma of a methoxypyrazine-free white wine, while approximately 15 ng/L affects red wine aroma (*3, 5*). The ability of a specific compound to impact the aroma of a wine depends in the first instance on the specificity of the associated aromatic note (*6*). In addition to the fact that the vegetable character of wine may primarily be attributed to the presence of IBMP, Escudero et al. have shown that other volatile compounds may synergistically interact with IBMP to significantly enhance the perceived pepper odor nuance in wine (*7*). In light of the above, it may be concluded that IBMP has a significant impact on the aroma of Sauvignon blanc wine when

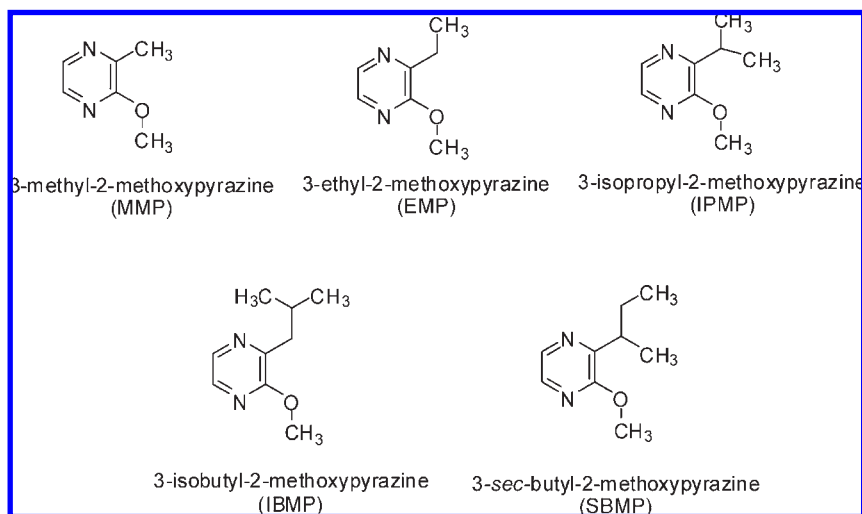*Corresponding author. Tel: (+27) 21 808 3351. Fax: (+27) 21 808 3360. E-mail: ajdevill@sun.ac.za.

Figure 1. Chemical structures of the five principal 3-alkyl-2-methoxypyrazines.

present at levels of the order of a few ng/L (parts per trillion or ppt). In fact, MP concentrations in the range of approximately 8 to 15 ng/L are considered to contribute constructively to the desirable herbaceous or vegetative aroma of this variety, whereas concentrations as high as 30 ng/L are often considered overpowering and out of balance (*8*).

MP concentrations in grapes, and by extension the produced wine, are influenced by a multiplicity of factors including grape variety, fruit maturity, season, climate and solar exposure of the fruit (*8, 9*). A previous study of 203 South African Sauvignon blanc wines reported IBMP values ranging from <1 to 14 ng/L (*9*). Australian Sauvignon blanc wines were found to contain approximately 1 to 15 ng/L IBMP, while concentrations in products from France and New Zealand typically range between 5 to 40 ng/L and 10 to 35 ng/L, respectively (*4*).

Due to their potentially important contribution to wine aroma, several analytical methodologies have been developed for the measurement of selected MPs in wine. These almost exclusively comprise gas chromatography (GC), either in conjunction with mass spectrometric (MS) (*4, 5, 10−12*) or nitrogen−phosphorus selective detection (NPD) (*13−15*). Due to the very low natural levels of these analytes, sample preconcentration is indispensable, and generally involves cleanup and preconcentration utilizing techniques such as liquid extraction (LE) (*4, 5, 10, 11*), distillation (*4, 5, 11, 14, 16*), solid phase extraction (SPE) (*4, 5, 11, 16*) and solid phase microextraction (SPME) (*12−15*). An internal standard is universally used for quantitation with the choice of internal standard varying between deuterium-labeled analogues (*4, 5, 11, 12*) and alternatively substituted pyrazines (*10, 14, 15*). Minimum detection levels achieved by GC vary between approximately 0.2 and 2 ng/L IBMP. These GC methods are therefore generally capable of accurate determination of IBMP in most wines, although the levels of IPMP and SBMP are often below the detection limit.

We have investigated liquid chromatography−mass spectrometry (LC−MS) as an alternative analytical technique for MP determination. Heymann et al. (*16*) have reported an HPLC−UV method for methoxypyrazine analysis, although minimum detection levels of 1.2 μg/L rendered this method entirely unsuitable for wine analysis. LC−MS was selected in the current study for a number of reasons. Most importantly, LC offers the advantages of higher sample loading capacity and superior sample introduction precision compared to GC. In addition, very high ionization efficiencies may be obtained on modern LC−MS instrumentation (*17*). Coupled to this is the fact that MPs contain

heteroatoms in the aromatic ring, as well as electron donating methoxy groups and alkyl side chains—structural characteristics that aid charge stabilization in positive ionization mode. Moreover, LC is inherently compatible with acids, which may be indispensable as part of an efficient sample preparation procedure for these compounds. Taken together, these factors suggest that LC−MS may provide very sensitive analysis of MPs, despite the fact that chromatographic efficiency is lower in LC compared to GC. The high degree of selectivity inherent to utilizing LC−MS/MS in multiple reaction monitoring (MRM) mode may then be used to compensate for the relatively low resolving power of LC, although this places stringent demands on sample cleanup. Combined with LC−MS/MS, distillation in combination with LE was selected for preconcentration of samples in this study, as this procedure offered quantitative preconcentration of analytes and is sufficiently robust to allow the analysis of large numbers of samples.

Here we report an optimized and validated LC−MS/MS method suitable for the trace-level determination of the three principal MPs in wine. Quantitative results obtained from the analyses of 575 South African Sauvignon blanc wines, as well as several additional varieties, will presented and discussed.

MATERIALS AND METHODS

Chemicals and Standards. Acetonitrile, dichloromethane (HPLC grade) and tannic acid (CP grade) were from Merck (Darmstadt, Germany). IBMP, IPMP, SBMP, methanol and ethanol (HPLC grade) were obtained from Aldrich (Aston Manor, South Africa), and formic acid (CP grade) was obtained from Associated Chemical Enterprises (Mulbarton, South Africa). Standards were prepared by weighing appropriate amounts of reference material on an analytical balance followed by dilution in A-grade volumetric glassware. Intermediate standards were prepared in ethanol while working standards were prepared in 10% ethanol.

A total of 575 South African Sauvignon blanc wines, from all wine producing regions and vintages 1999−2007, were analyzed. A limited number of wines of other cultivars (all recent vintages) were also analyzed. Samples were obtained from submissions under the South African controlled appellations system (South African Wine and Spirit Board) as well as from export applications (South African National Department of Agriculture). Additional chemical data for the Sauvignon blanc wines were obtained from archived data used for certification of the products under the South African controlled appellations system. Alcohol content was determined by densitometry, total reducing sugars by flow-injection analysis, total acidity by titration and pH using a pH meter.

Optimized Sample Preparation Procedure. To 500 mL of wine, a single glass ball and spatula tip of tannic acid (to prevent foaming) were

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added, and the sample was distilled utilizing a 60 cm fractionating column, continuing distillation until 100 mL of distillate was collected. The distillate was cooled in a two-stage process, first passing through a water-cooled condenser followed by passage through a slurry of ice and water before collection in a 100 mL volumetric flask. The distillate was transferred quantitatively to a 500 mL separating funnel containing a 35 mm egg-shaped magnet. The distillate was extracted with three portions (10 mL, 5 mL and 5 mL) of dichloromethane by rapid stirring for 10 min at a time. The combined dichloromethane fractions were transferred to a pear-shaped vessel with a 1.5 mL graduated stem containing 0.5 mL of concentrated formic acid (to render analytes ionic and nonvolatile). The acidified dichloromethane extracts were evaporated at room temperature under a stream of nitrogen gas until less than 0.5 mL of concentrate remained. Finally the extract was reconstituted to 1 mL using a solution of 40% acetonitrile in water, homogenized and transferred to a 1.8 mL amber vial for analysis.

Chromatographic Details. A Waters Alliance 2695 liquid chromatograph (Waters Corporation, Milford, MA) equipped with a Micromass Quattro Premier XE tandem quadrupole mass spectrometric detector (Micromass, Manchester, U.K.) was used for all experiments. Sample extracts were separated by reversed phase liquid chromatography utilizing a methanol and water gradient and a phenyl hexyl column (Phenomenex Luna, 250 × 4.6 mm, 3 μm, Torrance, CA) thermostated at 40 °C. The gradient started at 35% methanol and increased to 85% in 18 min, followed by a column cleanup step consisting of 95% methanol for 2.5 min. The flow was maintained at 1.0 mL/min throughout, and the re-equilibration time was 3.5 min. The total run time was 25 min, while the divert valve was used to direct the effluent to the detector only between time 14 and 18 min, the rest being vented to waste. Variable injection volumes were used ranging from 5 to 100 μL. Atmospheric pressure chemical ionization in the positive ion mode was performed using the following optimized parameters: corona current 4.4 μA, cone voltage 34 V, source and desolvation temperatures 150 and 200 °C, respectively. The mass spectrometer was operated in multiple reaction monitoring mode. Acquisition parameters are given in **Table 1**.

Data Analysis and Statistical Methods. Multivariate data analysis was performed utilizing Statistica (Statsoft Inc., OK, versions 7 and 8). Analysis of variance (Anova) was performed following "bootstrap" correction for non-Gaussian distribution of the data. Quantitative data was standardized to produce variables with 0 mean and 1 standard deviation for principal component analysis (PCA). In cases where the IPMP and SBMP content of the samples was below the LOQ (0.10 ng/L) of the method, samples were removed from the data set prior to PCA.

RESULTS AND DISCUSSION

Method Development. The objective of this study was the determination of three MPs in South African Sauvignon blanc wines at their natural levels of occurrence. As the concentrations of MPs in Sauvignon blanc wines are in the sub- to low parts per trillion range, it is essential that appropriate sample preparation and analysis methods be developed that are capable of precise and accurate measurements of these components in wine. In order to attain these low detection limits, the large sample loading capacity of LC was exploited in the current study, which necessitated highly concentrated sample extracts. Considering that method sensitivity is directly related to the level of sample preconcentration achieved, a labor-intensive sample preparation strategy is unavoidable.

Various sample preparation strategies were evaluated for this purpose, including combinations of the following: distillation, SPE, stir bar sorptive extraction in combination with liquid desorption (SBSE-LD) and LE. SPE using C18, polystyrene-divinylbenzene (PSDVB) and polymeric mixed mode chemistries in particular displayed adequate selectivity for the components of interest. Although this allowed efficient sample cleanup and preconcentration, optimized procedures generally lacked the capacity to attain the required preconcentration factors (500×)

Table 1. Parameters for Acquisition of MRM Data

compound	parent ion (Da)	collision energy (eV)	1° daughter ion (Da)	collision energy (eV)	2° daughter ion (Da)
IPMP	152.9	18	137.9	26	122.9
IBMP	167.0	16	125.0	22	124.0
SBMP	167.0	18	138.0	24	123.0

demanded by the current application and in addition would require impractical sample volumes (on the order of 250 mL) to provide sufficient sample volumes (0.5 mL).

In contrast, distillation of wine samples as the first step in the sample preparation procedure ensured the effective isolation of volatile constituents and served to reduce the sample volume for subsequent steps. The fact that the nonvolatile components were eliminated in this step contributed significantly to the robustness of the overall method as this effectively prevented any buildup of deposits in the ionization source or column. Subsequently, three solvents were evaluated for liquid extraction of the distillates, namely, diethyl ether, dichloromethane and hexane. Dichloromethane provided the highest extraction efficiencies from the aqueous alcoholic phase (i.e., distillates of wine samples). In addition, the higher density of dichloromethane makes this solvent compatible with multiple extractions using a separating funnel, while its high vapor pressure facilitates removal by evaporation.

Compared to sample preparation methods reported in the literature for MP determination, this procedure requires much higher volumes of wine, while similar sample throughput is achieved compared to the most sensitive GC−MS and GC−NPD methods reported in the literature (*5*, *11*, *14*, *15*). Nevertheless, the relatively lengthy sample pretreatment procedure utilized here is fully justified by very low detection levels attained (5−66 times lower than published GC−MS or GC−NPD methods). Moreover, the very good recovery and precision achieved using this method (see validation results below) obviates the requirement for internal standards for quantitation, as is generally the case with GC methods. It should further be noted that the acidic nature of sample extracts obtained with this strategy renders these extracts incompatible with GC separation.

Initially, both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were evaluated for the analysis of MPs. ESI provided more efficient ionization of the target species, and limits of detection were approximately an order of magnitude lower compared to APCI. However, analysis of normal and spiked wine extracts consistently showed severe matrix quenching effects for ESI. Attempts to alleviate this by varying ionization parameters and adding additional sample cleanup steps involving SPE proved ineffective. Off-line analysis of collected fractions of extracts using GC−MS and LC−MS in an effort to identify interfering matrix components were equally unsuccessful.

Since the detector response in APCI mode was largely independent of matrix interference, optimization of APCI conditions was performed, and this ionization mode was subsequently used exclusively. Optimal ionization conditions are outlined in Materials and Methods.

Various reversed phase column chemistries were evaluated for separation of sample extracts, including CN, C5, C8, C18, and phenyl hexyl phases (*18*). The objective was to identify the phase that offered the strongest retention of the analytes, allowing their elution using a mobile phase with the highest organic content for optimal ionization efficiency. In addition, the selectivity of each phase was evaluated in terms of achieved separation between analyte peaks and matrix components detected in the extracts in
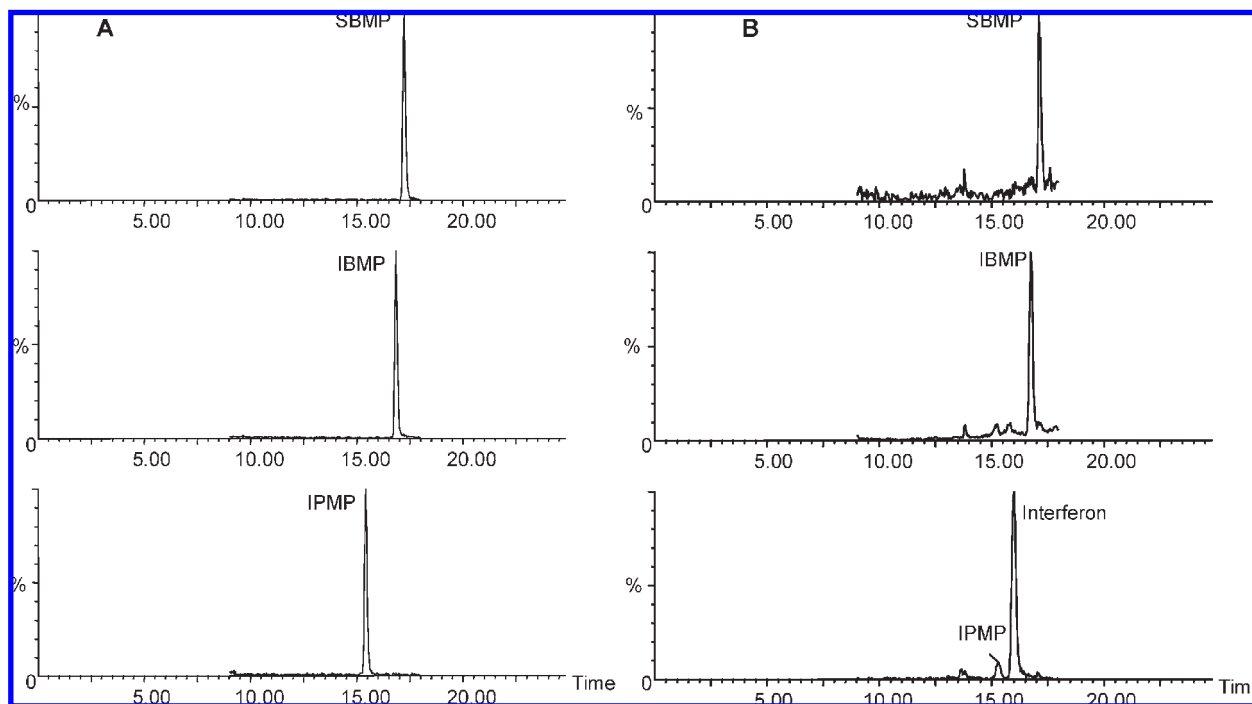
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Figure 2. Chromatograms obtained for the LC−APCI-MS/MS analysis of (**A**) a standard solution, 60.4 pg of SBMP, 238.4 pg of IBMP and 61.6 pg of IPMP on column, and (**B**) an extract of a Sauvignon blanc wine, concentration factor 500, 100 μL injected. 12.0 pg of SBMP, 545.0 pg of IBMP and 31.5 pg of IPMP on column. Retention times: IPMP 15.32 min, IBMP 16.75 min and SBMP 17.14 min. Only the primary transitions are shown in each case.

MRM mode. The phenyl hexyl phase was found to produce the best results according to both criteria.

The initial objective of the study was to determine five MPs (IBMP, SBMP, IPMP, EMP and MMP) in wine. However, under optimal experimental conditions using the phenyl hexyl phase, EMP and MMP elute relatively early in the gradient separation (i.e., in a mobile phase that has a relatively high aqueous content) and therefore their ionization was less efficient. These early eluting compounds also possibly coelute together with more matrix components. This phenomenon was exacerbated by virtue of their relatively lower molar masses, increasing the probability of interference by coeluting species in MRM mode. Taken together, these factors rendered the detection levels for EMP and MMP too high for the purpose of their determination in wine. For this reason, subsequent experiments focused exclusively on the principal three MPs.

Figure 2 presents MRM chromatograms obtained for a standard solution and a Sauvignon blanc wine extract utilizing the optimized LC−MS/MS method (only primary ion transitions are shown). It is important to note that IPMP is separated from an interfering compound in the wine extract and detected in MRM mode using the phenyl hexyl column.

Method Validation. The optimized analytical procedure utilizing distillation and solvent extraction followed by LC−APCI-MS/MS analysis was validated to ensure its suitability for routine analysis. Parameters included determination of analyte recovery from Sauvignon blanc wine matrices, linear operating range, sensitivity (LODs and LOQs), specificity, accuracy, precision and system stability.

The recovery study involved nine replicate sets of measurements performed at three levels of fortification, 1, 10, and 100 ng/L. Fortified samples were prepared by adding appropriate amounts of reference standards to Sauvignon blanc wine prior to extraction. Analyte recoveries were determined by subtracting values obtained for the unfortified wine matrix at each level of fortification. Analyte recoveries for the analytical procedure were

Table 2. Results for the Recovery Study at Three Levels of Fortification (1, 10, and 100 ng/L)

fortification level	recovery (% RSD)		
	IPMP	IBMP	SBMP
1 ng/L, $n = 9$	95.1 (7.1%)	100.7 (10.3%)	93.5 (5.8%)
10 ng/L, $n = 9$	90.8 (11.6%)	97.3 (6.0%)	94.4 (5.7%)
100 ng/L, $n = 9$	89.3 (6.5%)	96.1 (4.7%)	93.6 (4.3%)
overall, $n = 27$	91.8 (8.8%)	98.0 (7.5%)	93.8 (5.1%)

above 90% with RSD values below 9% ($n = 27$, 3 concentration levels). A summary of the results obtained for the recovery study is presented in **Table 2**. The LOD and LOQ values of the method (based on 3 and 10 times the signal-to-noise ratio) were 0.03 ng/L and 0.10 ng/L, respectively, for all three analytes. The method therefore offers enhanced sensitivity compared to other techniques used for the same purpose. IBMP was quantified in all Sauvignon blanc samples, while IPMP and SBMP were quantified in the majority of the samples. Linear system response was demonstrated from 5 to 4000 pg on column for IBMP and 5 to 1000 pg for IPMP and SBMP ($r^2 > 0.9999$ for primary and secondary ion transitions). Calibration was performed between 20 and 1000 pg (IBMP) or 10 and 500 pg (IPMP and SBMP) injected on column. For 4% of the analyzed samples, IBMP levels were outside the calibration range for injection volumes of 100 μL—in these instances the extracts were reanalyzed using suitable injection volumes. Method specificity for compounds of interest was based on retention time and the ratio of abundance of the relevant primary and secondary ions. RSD values below 0.3% and 8.2% were measured for retention times and ion ratios, respectively ($n = 36$, using fortified wine samples). These values fall well within accepted criteria (*19*). Instrument precision, as determined by reproducibility for repeated injections of standard solutions, was better than 4.4% ($n = 7$). The overall method precision is therefore sufficient to perform a single determination per sample. Finally, the long-term stability of the system was

evaluated upon completion of the investigation by comparing the response for standards used in the routine calibration of the instrument. The system response was constant, as indicated by a RSD value of 6.9% for the peak area of a calibration standard during the three-month period that the samples were analyzed. In this period the system was calibrated 16 times and approximately 700 wine samples were analyzed.

Quantitative Results for South African Sauvignon Blanc Wines. A total of 575 South African Sauvignon blanc wine samples from all wine-producing regions of South Africa and spanning vintages from 1999 to 2007 were analyzed. Due to sample availability constraints, 82% of the samples were of the 2004 and 2005 vintages. The vintage distribution of the samples was as follows (numbers of samples appear in parentheses): 1999 (1), 2000 (1), 2001 (1), 2002 (10), 2003 (53), 2004 (189), 2005 (281), 2006 (36) and 2007 (3). South African Sauvignon blanc wines are mainly produced in the Breede River Valley and Coastal regions. Since 118 and 268 samples were from these respective regions, sampling may be considered reasonably representative regarding geographical origin. Measured values for IBMP ranged between 0.40 and 44 ng/L, with an average of 6.2 ng/L and median of 4.3 ng/L, indicating that the distribution of values is not symmetrical: the majority of the samples were at the lower end of the scale (**Figure 3**). The highest number of samples contain between < 1 and 5 ng/L IBMP. The concentrations of IPMP and SBMP were consistently lower than those of IBMP, and ranged from < 0.03 to 3.9 ng/L and < 0.03 to 3.2 ng/L, respectively. The ratios of IPMP to IBMP and SBMP to IBMP were approximately 10% in each case. These ratios displayed less variation than the overall IBMP concentration, a phenomenon that was also observed by
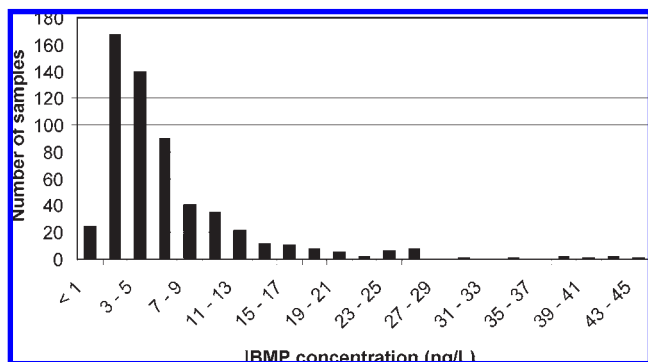
Lacey et al. (*4*). The variation in the ratio of IPMP to IBMP was 46% and that of SBMP to IBMP 67%, whereas the overall IBMP concentration displayed a variance of 102%.

It is evident from the data that it certainly is possible to produce Sauvignon blanc wine in South Africa that possess the desired and characteristic herbaceous aroma. A total of 82% of the samples contained IBMP above the perceptible level in white wine (2 ng/L), while 16% of the products contained more that 10 ng/L IBMP. Allan et al. (*8*) reported that there is a narrow concentration window of total 3-alkyl-2-methoxypyrazines that allows for the desirable methoxypyrazine flavor contribution to be evident yet not excessive. Recognition of the herbaceous nuance occurred at approximately 4 to 8 ng/L, while the desirable range is described as ranging between 8 and 15 ng/L of total 3-alkyl-2-methoxypyrazines. Concentrations as high as 30 ng/L are often considered to be overpowering and out of balance.

Table 3 presents a summary of the IBMP content of vintages 2002 to 2006 for wines grouped according to geographical origin. Maximum values for IBMP show some variance (17 to 44 ng/L) across the wine producing regions and over the sampled vintages. Minimum, maximum and average values obtained for those samples for which a specific regional delimitation was available are graphically depicted in **Figure 4**.

It has been hypothesized that region of origin plays an important role in determining 3-alkyl-2-methoxypyrazine content of Sauvignon blanc wines. Quantitative differences have been reported between French, New Zealand and Australian products (*4*). Local investigations attributed regional differences in

Figure 3. Distribution of IBMP levels in 575 South African Sauvignon blanc wines as determined using LC−MS.
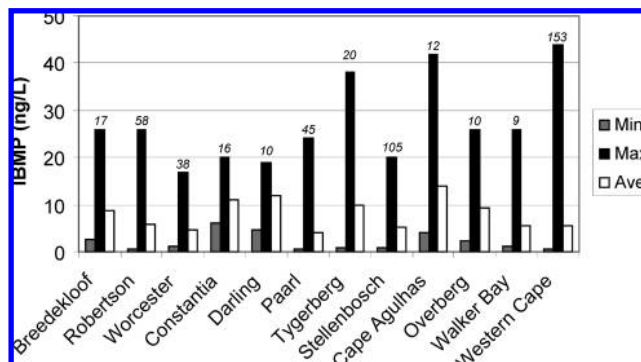

Figure 4. Summary of the minimum, maximum and average values of IBMP in Sauvignon blanc wine as a function of region. Only samples with specific regional classification are shown, vintages 1999 to 2007. Number of samples appears above graphs for each region.
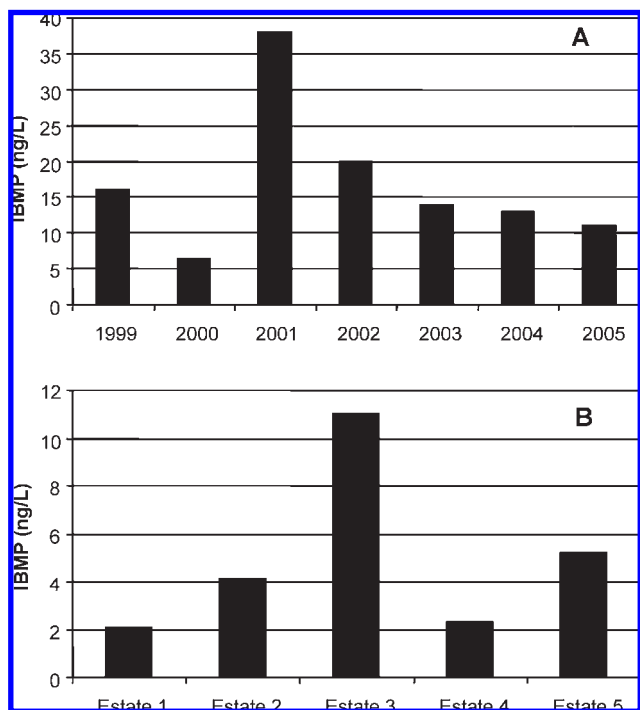
Table 3. IBMP Levels (ng/L) in Sauvignon Blanc Wines from Different Wine-Making Regions over Separate Vintages of 2002−2006 (Minimum−Maximum/Average (Number of Samples))

region/district	2002	2003	2004	2005	2006
Breede River Valley					
Breedekloof		2.6−20/7.0 (10)	4.4−26/11 (7)		
Robertson			0.66−26/6.0 (51)		2.2−13/5.8 (4)
Worcester	1.8−16/6.6 (3)	1.3−11/3.4 (17)	2.8−17/5.4 (18)		
Coastal Region					
Constantia			12−18/15 (3)	6.6−20/11 (12)	
Darling				13- 19/15 (6)	
Paarl			0.82−7.8/3.6 (16)	0.52−24/4.0 (26)	
Tygerberg		0.74−14/5.2 (5)	3.6−13/8.8 (4)	6.4- 12/9.6 (7)	
Stellenbosch		3.4−5.6/4.4 (4)	0.76−20/4.8 (30)	1.0−16/5.4 (66)	2.8−10/5.4 (3)
Cape Agulhas				4.2−14/9.6 (10)	
Overberg			2.4−26/8.6 (4)	5.6−13/9.6 (4)	
Walker Bay				1.2−8.4/3.4 (7)	
Western Cape[a]		1.2−7.0/3.4 (4)	1.7−9.8/5.0 (33)	0.48−44/5.0 (102)	0.58−24/9.4 (12)

[a] All wine producing areas of the Western Cape.

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Figure 5. (**A**) IBMP content of Sauvignon blanc wines produced on the same estate over seven consecutive vintages (1999 to 2005). (**B**) IBMP content of Sauvignon wines (Stellenbosch, 2004 vintage) produced on five different estates located in close proximity.

IBMP concentration to macro- and microclimatic variations between regions and seasons (*20*). From the information in **Figure 4** it can be observed that some regions such as Constantia and Darling, which are distinguished by cooler climates due to their close proximity to the ocean, are characterized by relatively high average IBMP values. Maximum IBMP values of these regions are nevertheless lower than those of some warmer (inland) regions such as Robertson and Paarl, which in turn had lower average values. On the other hand, Cape Agulhas, a cool region in close proximity to the ocean, displays a relatively high average and maximum value. This variable nature in levels of occurrence of IBMP in South African Sauvignon blanc wines may be illustrated at the hand of two examples. **Figure 5A** presents an illustration of the IBMP content of wines produced on the same estate in the Tygerberg district for seven consecutive vintages (1999 to 2005). The IBMP content varies between 6.4 and 38 ng/L (60% RSD). Significant variability of IBMP levels is also noted for wines that were produced during the 2004 vintage in the Stellenbosch district, on five adjacent estates, located approximately 8 km apart (**Figure 5B**). IBMP concentrations ranging from 2.1 to 11 ng/L were measured (72% RSD). This information suggests that there is no clear relation between the IBMP content of South African Sauvignon blanc wines and origin or vintage. IPMP and SBMP levels followed similar trends to those mentioned for IBMP.

To further investigate possible correlations between geographical origin and vintage with MP content, statistical investigation of the quantitative data was undertaken. Anova was used to elucidate significant differences in MP content of South African Sauvignon blanc wine as a function of vintage and geographical origin. However, Anova requires a Gaussian distribution of values and an equal representation within different groups (i.e., vintages or geographical origin). Due to sample availability constraints these requirements were not fulfilled in all instances. Bootstrap correction was therefore applied prior to performing

Anova. **Figure 6A** shows the means and 95% confidence intervals (corrected) for IBMP concentrations from different regions. The results indicate that significant differences ($p < 0.05$) in MP content exist between wines of certain origins (e.g., between Stellenbosch and Overberg, Cape Agulhas, Constantia and Darling). Similar trends were observed for IPMP and SBMP (results not shown). Moreover, significant differences in IBMP content between vintages ($p < 0.05$) were observed (**Figure 6B**). These results are slightly misleading, however, as only vintages 2004 and 2005 represent sufficient sample numbers (189 and 281 samples, respectively) to allow accurate conclusions to be drawn. No significant differences in IBMP content were evident between these vintages. The same trends were also observed for IPMP and SBMP.

The multivariate data analysis technique principal component analysis (PCA) was used to determine correlations between properties of the wines. Initial screening of different regions using PCA also showed no clear grouping according to geographical origin. We therefore focused on wines from Constantia and Worcester only as these represent cool and warm regions, respectively, so that more pronounced differences in the MP content are expected. Wines of vintages 2002 to 2005 (Constantia) and 2002 to 2004 (Worcester) and levels of the three quantified MPs were used for this purpose. Scores and loadings plots for the methoxypyrazine data from these two regions are presented in **Figure 7**. In the loadings plot, PC1 accounts for 86% of the total variance, and the content of all three MPs is highly (negatively) correlated with this PC. The scores plot shows that the predominance of Constantia wines along the axis of the first PC can be ascribed to higher levels of all MPs in these wines, as is expected for cooler climate wines. The second PC seems to reflect different ratios of MPs, with some Worcester wines in particular showing high scores on this PC. However, despite these observations, clear differentiation is not achieved as some Constantia wines are not distinguished from Worcester wines on the first 3 PCs. Addition of wine chemical data (alcohol and sugar content, pH and total acidity) did not improve this differentiation. It is interesting to note, though, that these parameters primarily contribute to PC2, and display very low covariance with MP content. General wine data such as sugar and alcohol content may be considered indicators of climatological factors—warmer climates generally produce higher sugar levels and hence lead to the production of more alcoholic wines. These wines are also expected to contain lower concentrations of MPs. The lack of covariance between these parameters is therefore rather surprising.

Roujou de Boubee et al. reported correlation between the breakdown of malic acid and IBMP in grapes during ripening, suggesting that these parameters are similar indicators of grape ripeness (*5*). Our data reveal no correlation between the total acidity or sugar content of bottled wines and MP content. Relatively high total acidity and/or sugar content with concurrently high MP levels may possibly indicate that the grapes were harvested before optimal ripeness, a common practice in warmer climates to attain higher vegetative flavors. Any potential correlation between total acidity, sugar content and MP levels is presumably further obscured by variations in fermentation practices for the studied wines.

Vintage has been reported to play an important role in determining the MP content of wines from a particular region, mainly through annual variations in average temperature and rainfall. This aspect was investigated using data for the Stellenbosch region only, as sufficient samples for each vintage were available from this district, and to remove additional variability ascribed to region of origin. The scores and loadings plots for PC1 vs PC2 for these wines are presented in **Figure 8**. Wines with
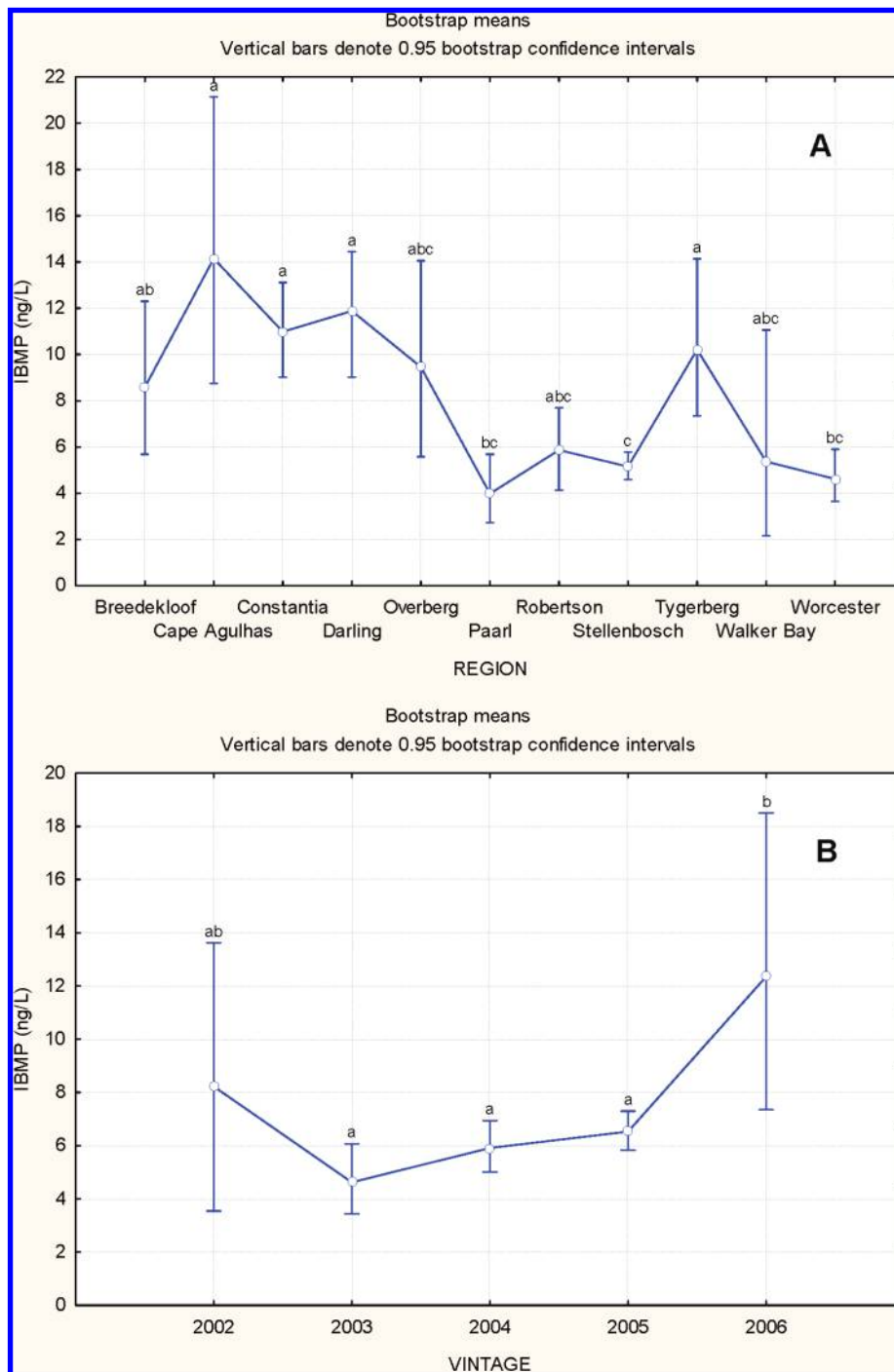
Figure 6. Anova results for IBMP concentrations in Sauvignon blanc wines (means and 95% confidence intervals, bootstrap corrected). (**A**) According to regions, and (**B**) according to vintage.

higher total MP content are partially separated on the left of the scores plot along PC1, which accounts for 80% of the variance. PC1 displays high negative loadings for all three MPs. Some differentiation of wine samples is also observed along PC2 (15% of the variance), which may possibly be ascribed to differences in SBMP content in these wines (this compounds displays a relatively high negative loading on PC2). However, there is no clear differentiation according to vintage in this figure, or indeed in any of the three PCs. As before, incorporation of additional wine parameters did not affect these conclusions.

These data therefore suggest that the factors region of origin and vintage are not primarily determinative regarding the MP content. The regional data in **Table 3** and **Figure 4** seem to

reinforce this observation as there is clearly no obvious correlation between maximum and average values between the regions, which would indicate that a particular region produces consistently high values. The significance of this is that although a particular region may possess a low average, a relatively high maximum value may at the same time be expected, a point that is well illustrated by the data for the Paarl region (**Figure 4**). Cape Agulhas is an exception, producing simultaneously a high average and high maximum value, although this might be explained by the relatively small number of wines analyzed from this region.

Unsuccessful differentiation of South African Sauvignon blanc wine may therefore principally be attributed to the wide variation in the MP content of the samples, a phenomenon reported by
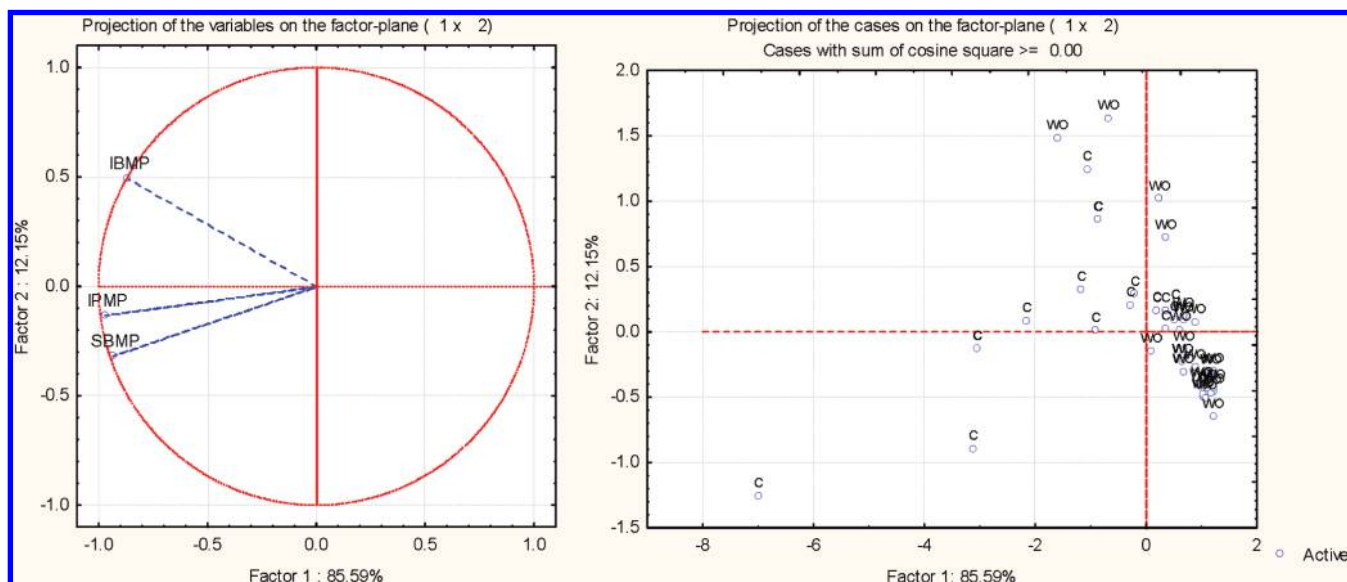
Figure 7. PCA loading and scores plots for Sauvignon blanc samples from samples from Constantia (C) and Worcester (WO) obtained using methoxypyrazine data (PC1 versus PC2, denoted Factor 1 and Factor 2 in Statistica Software).
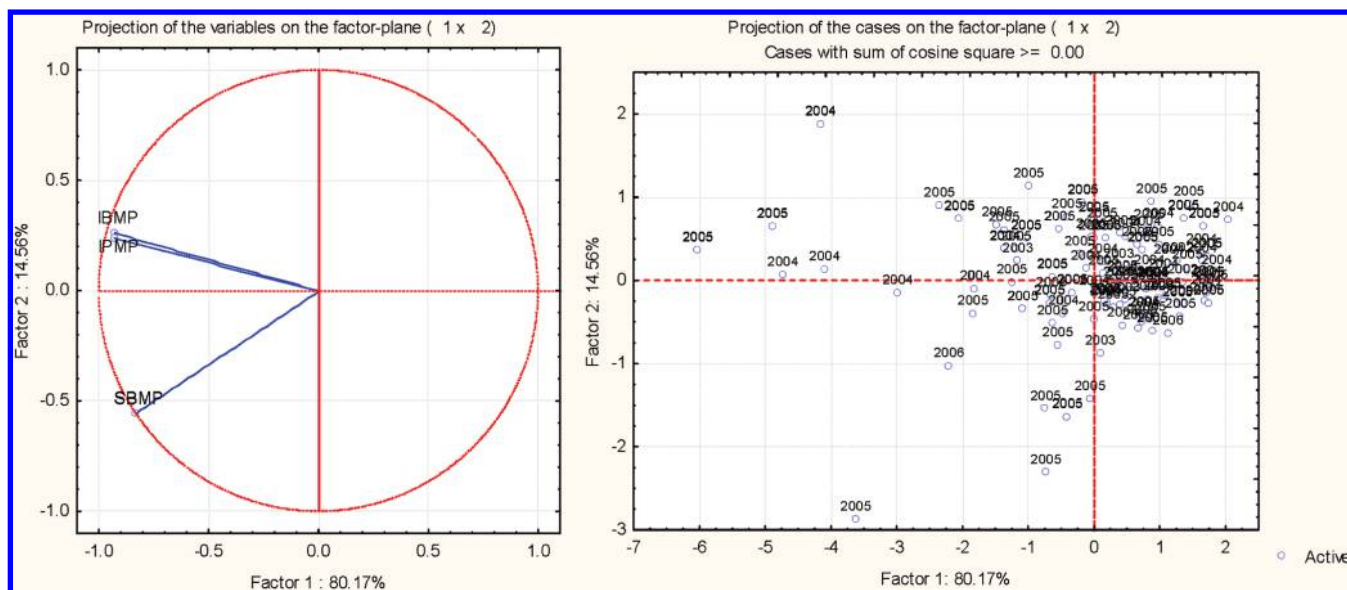

Figure 8. PCA loading and scores plots for Sauvignon blanc samples of different vintages from the Stellenbosch region obtained using methoxypyrazine data (PC1 versus PC2, denoted Factor 1 and Factor 2 in Statistica Software).

various other authors (*4*, *5*, *9*, *21*, *22*). It is known that the concentration of these components in grapes declines dramatically during ripening. Factors associated with this decline include ripening temperature, viticultural conditions (soil type, pruning, training and plantation density) and light exposure (*4,5,20−22*). Production variables (skin contact and settling) have been reported to have an equally dramatic effect on the MP content of must and also play a role in determining the final concentration (*23, 24*). It may therefore be assumed that all these factors act together in a complex manner so that the final MP concentration of the samples is not simply the product of vintage or origin but rather an intricate combination of diverse factors.

Quantitative Results for Other Wine Cultivars. The developed LC−MS/MS method was also applied to the analysis of MPs in a limited number of South African wines of other cultivars. The results for two additional white and three red wine varieties are presented in **Table 4**. Only IBMP was quantified in these wines

Table 4. IBMP Levels for Five Different South African Wine Cultivars

cultivar	no. of samples	min (ng/L)	max (ng/L)	av (ng/L)	RSD (%)
Pinotage	6	0.78	3.1	1.5	57
Chardonnay	6	0.20	1.0	0.53	53
Cabernet Sauvignon	6	3.4	17	10	48
Shiraz	6	1.5	3.4	2.4	26
Chenin blanc	4	0.38	1.1	0.59	59

since SBMP and IPMP were present at levels beneath the detection limits of the method. The ratios of the latter two compounds relative to IBMP were less than 10%, as also observed by Allen et al. (*11*). Based on the limited quantitative data presented in **Table 4**, it is evident that MPs are expected to contribute little to the aroma of all of these cultivars, with the

exception of Cabernet Sauvignon. As was the case for South African Sauvignon blanc wines, IBMP concentrations in Cabernet Sauvignon were slightly lower than in French products, for which an average value of 18 ng/L has been reported (*5*). Hashizume and Samuta reported low levels (∼2 ng/kg) of IBMP in ripe Chardonnay grapes (*22*), but we were unable to obtain literature references reporting MP levels in wines of any of the other cultivars. This is presumably at least partially due to the levels being below LODs of most GC methods. Pinotage is a uniquely South African cultivar, and to the best of our knowledge this represents the first report of IBMP in either grapes or wine of this cultivar. Extension of the number of wine samples analyzed using the current method might well shed more light on the potential aroma contribution of MPs in non-Sauvignon blanc cultivars.

ABBREVIATIONS USED

IBMP, 3-isobutyl-2-methoxypyrazine; IPMP, 3-isopropyl-2-methoxypyrazine; SBMP, 3-*sec*-butyl-2-methoxypyrazine; MP, methoxypyrazine; LOD, limit of detection; LOQ, limit of quantitation; GC, gas chromatography; GC−NPD, gas chromatography nitrogen−phosphorus detection; HPLC−UV, high performance liquid chromatography−ultraviolet detection; LC, liquid chromatography; LC−MS, liquid chromatography−mass spectrometry; GC−MS, gas chromatography−mass spectrometry; SPME, solid phase microextraction; SPE, solid phase extraction; LE, liquid extraction; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; MRM, multiple reaction monitoring; m/z, mass to charge ratio; PCA, principle component analysis; PC1, first principal component; Anova, analysis of variance; RSD, relative standard deviation.

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