

Quantitative Survey of 3-alkyl-2-methoxypyrazines and First Confirmation of 3-ethyl-2-methoxypyrazine in South African Sauvignon blanc Wines

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3-Alkyl-2-methoxypyrazines are important aroma components in Sauvignon blanc wine, contributing to the typical vegetative character associated with the cultivar. The analysis of methoxypyrazines is highly challenging and, as a result, limited quantitative data are available for South African Sauvignon blanc wines. In this study, liquid chromatography-tandem mass spectrometry was applied for the sensitive quantification of the three principal methoxypyrazines in 881 South African Sauvignon blanc wines. The total wine methoxypyrazine concentration exceeded the combined recognition threshold for the vegetative aroma in more than 60% of the samples, demonstrating the involvement of these compounds in the typical aroma of the cultivar. The analytical method was adapted to allow the accurate quantitation and spectral confirmation of 3-ethyl-2-methoxypyrazine in Sauvignon blanc wines for the first time. Concentrations ranged between 1.1 and 4.9 ng/L. Statistically significant relationships between wine methoxypyrazine concentrations and parameters related to the maturity of the grapes and to the climate, as well as geographical origin and vintage, were found.

INTRODUCTION

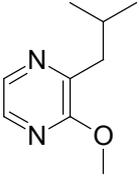
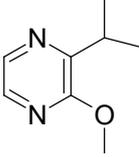
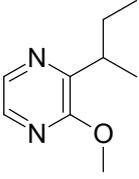
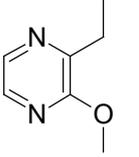
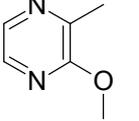
Sauvignon blanc is a distinctive dry white wine with a characteristic aroma described as nuances of vegetative, herbaceous and green pepper. Three 3-alkyl-2-methoxypyrazines (MPs), namely 3-isobutyl-methoxypyrazine (IBMP), 3-*sec*-butyl-methoxypyrazine (SBMP) and 3-isopropyl-2-methoxypyrazine (IPMP), are principally responsible for the distinctive vegetal character of the cultivar (Lacey *et al.*, 1991; Allen & Lacey, 1998; Roujou de Boubee *et al.*, 2000; Sala *et al.*, 2002; Marais, 2004; Marais *et al.*, 2004). These grape-derived flavour components have extremely low sensory detection thresholds (Table 1). Some congeners influence the aroma of white wine at concentrations of 1 ng/L to 2 ng/L, while their detection threshold in red wine is 10 ng/L to 15 ng/L (Allen & Lacey, 1998; Roujou de Boubee *et al.*, 2000; Marais *et al.*, 2004). In wine, MPs typically occur at trace level amounts, with a combined concentration that is in the order of 1 ng/L to 40 ng/L (Allen & Lacey, 1998).

IBMP is invariably the most abundant congener found in Sauvignon blanc wine, representing approximately 80% of the total, while IPMP and SBMP occur in roughly equal amounts (Allen *et al.*, 1991; Marais *et al.*, 2004; Alberts *et al.*, 2009). The occurrence of 3-ethyl-2-methoxypyrazine (EMP) in wine has also been reported tentatively (Augustyn *et al.*, 1982; Allen & Lacey, 1998; Sala *et al.*, 2000, 2002), while the possible occurrence of 3-methyl-2-methoxypyrazine (MMP) has been suggested on the basis of a feasible biosynthetic route (Lacey *et al.*, 1991). These latter pyrazines are associated with “earthy” aromas and have relatively high olfactory detection thresholds (Table 1); they therefore are unlikely to contribute significantly to the typical “green” Sauvignon blanc wine aroma. Sala *et al.* (2000, 2002) reported the presence of EMP in Merlot and Cabernet Sauvignon musts and wines, but found concentrations that were consistently below the quantification level of their analytical methods (0.5 ng/L to 4 ng/L). In a preliminary investigation, Allen and Lacey (1998) reported levels of EMP as high as 1 000

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TABLE 1

Chemical structures, odour thresholds and flavour properties of the principal 3-alkyl-2-methoxypyrazines found in wine.

Compound	Structure	Odour threshold in water (ng/L) ^a	Flavour description ^b
IBMP		1 - 2	Bell peppers
IPMP		1 - 2	Bell peppers, green peas
SBMP		1 - 2	Galbanum, ivy leaves, green peas
EMP		425	Raw potato
MMP		4 000	Roasted peanuts

^aAllen *et al.*, 1998, Maga *et al.*, 1973^bMaga *et al.*, 1973, Murray *et al.*, 1975

ng/L in a Cabernet Sauvignon wine, as well as more than 100 ng/L in a Pinot noir wine. However, there is limited information available pertaining to the occurrence of this compound in grapes and wines of different cultivars. Apart from the single tentative report by Allen and Lacey (1998), who found unusually high levels of EMP, no quantitative data on EMP have been reported. This lack of information is largely due to the challenges associated with the analysis of these trace level compounds in the complex wine matrix (Schmarr *et al.*, 2010).

In grapes, the concentrations of MPs are strongly influenced by climatic and viticultural conditions, and generally decrease after *véraison* (Allen & Lacey, 1998; Roujou de Boubee *et al.*, 2000; Marais *et al.*, 2004). In addition, oenological factors also affect wine MP levels. As a consequence, Sauvignon blanc wine possessing the typical green pepper or vegetative aroma is sensitive to production factors such as geographical origin (Lacey *et al.*, 1991; Marais *et al.*, 1999), mesoclimatic conditions (Lacey *et al.*, 1991; Marais *et al.*, 1999; Falcão *et al.*, 2007), canopy microclimate (Marais *et al.*, 1999; Sala *et al.*, 2005), water availability (Sala *et al.*, 2005), solar radiation (Hashizume & Samuta *et al.*, 1999; Sala *et al.*, 2004) and time of harvesting (Ryona *et al.*, 2010). Marais *et al.* (1999) reported that canopy microclimate plays a particularly important role

in determining grape MP levels and that exposure to solar radiation, as manipulated by canopy management, has a more prominent effect than climatic temperature alone. Viticultural practices (Marais *et al.*, 1999; Maggu *et al.*, 2007) and the yeast strain used (Pickering *et al.*, 2008) also affect wine MP concentrations. Moreover, possible infestations with lady beetles may also contribute MPs to affected wines. In particular, elevated levels of IPMP and SBMP are associated with this phenomenon (Galvan *et al.*, 2008). It has also been reported that bottle closure and packaging type affect MP concentrations during wine storage, with the three major MPs affected to differing degrees. Some closure and packaging types even lead to increases in wine IPMP and SBMP concentrations (Blake *et al.*, 2009). This sensitivity of MP levels to oenological, production and ageing variables possibly contributes to the observed variance in their occurrence in Sauvignon blanc wine.

We have previously reported a survey on MP levels in South African Sauvignon blanc wines and found levels of IBMP ranging from 0.40 to 44 ng/L (RSD of 102%) in 577 wines, while concentrations of IPMP and SBMP ranged from < 0.03 to 3.9 ng/L and < 0.03 to 3.2 ng/L respectively (Alberts *et al.*, 2009). In the current study, 304 additional Sauvignon blanc wines were analysed. Data representing a broader span of vintages and origins were obtained with

the aim of improving the statistical representation of the South African Sauvignon blanc industry in order to allow closer investigation of trends in the data. Malic acid levels were also determined for a subset of samples, since malic acid concentration is strongly influenced by viticultural variables and therefore can reveal information pertaining to the climate of cultivation and the maturity of the grapes. Malic acid concentration was used together with general wine parameters, such as pH, alcohol content (Alc), total acidity (TA) and volatile acidity (VA), in an attempt to identify the factors that are responsible for the production of varietal-typical Sauvignon blanc wines possessing the vegetative aroma.

Moreover, given the limited information on MMP and EMP levels in Sauvignon blanc wines, the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method that was previously reported (Alberts *et al.*, 2009) was adapted to yield firm spectral information for the confirmation of the presence of EMP in these wines, as well as quantitative data on this compound in South African wines for the first time. This method utilises advantages inherent to LC-MS/MS to obtain very low detection limits.

MATERIALS AND METHODS

Chemicals and standards

High-purity chemicals and solvents were used throughout. Methanol, ethyl acetate, MMP, EMP, IPMP, IBMP and SBMP were obtained from Sigma-Aldrich (Mulbarton, South Africa); formic acid, malic acid, phosphoric acid and tannic acid were obtained from Saarchem (Wadeville, South Africa); while acetonitrile, dichloromethane and hexane were obtained from Burdick & Jackson (Honeywell International, Muskegon, USA).

Standards were prepared by weighing an appropriate amount of reference material on an analytical balance. All dilutions were made using A-grade glassware. Standards were prepared in a solution of 40% acetonitrile. Wine samples for the recovery study were fortified volumetrically by adding appropriate amounts of working standards.

Samples

A total of 881 South African Sauvignon blanc wines, from all wine-producing regions and of vintages 1999 to 2011, were analysed. The vintage distribution of these samples was as follows (number of samples in parenthesis) 1999 (1), 2000 (1), 2001 (1), 2002 (12), 2003 (56), 2004 (199), 2005 (308), 2006 (58), 2007 (54), 2008 (109), 2009 (49), 2010 (15) and 2011 (18). 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). General wine parameters such as pH, alcohol content, total acids and volatile acids were obtained from archived data that were used for the certification of these products under the South African controlled appellations system. Malic acid concentrations were determined for a subset ($n = 158$) of Sauvignon blanc wine samples using a liquid chromatography-ultraviolet detection (LC-UV) method as outlined below.

Sample preparation

Extraction of MPs for quantitative analysis

The procedure used for the analysis of MPs has been described previously (Alberts *et al.*, 2009). Briefly, 500 mL wine was distilled utilising a 60 cm fractionating column, and the first 100 mL of distillate was collected. The distillate was then extracted exhaustively with dichloromethane (10 mL, 5 mL and 5 mL, respectively). The combined dichloromethane fractions were acidified and concentrated by evaporation. Finally, the extract was reconstituted to 1 mL with a solution of 40% acetonitrile in water, homogenised, and transferred to a 1.8 mL vial for analysis.

Extraction of MMP and EMP for qualitative analysis

For spectral identification of MMP and EMP in the Sauvignon blanc extracts, further semi-quantitative concentration and purification were performed using the following procedure. Approximately 0.9 mL of extract was removed from the autosampler vial and transferred to a 5 mL test tube. A 1 mL aliquot of hexane was added, followed by extraction on a vortex mixer at high speed for one minute. The hexane phase was then drawn off and transferred to a 5 mL test tube containing 1 mL of 10% formic acid in ethyl acetate. This solution was homogenised on a vortex mixer and evaporated under a gentle stream of nitrogen at room temperature to a volume of $< 100 \mu\text{L}$. This residue was then redissolved in 200 μL of a solution of 50% acetonitrile in water. If required, acetonitrile was added drop-wise until a homogeneous solution was obtained following high-speed vortex mixing. Finally, the solution (*ca.* 300 μL) was transferred to a total-recovery autosampler vial for analysis.

Chromatographic details

LC-MS/MS methods for the determination of MPs

Quantitative analysis of MPs (HPLC program A)

Chromatographic analysis was performed as reported previously (Alberts *et al.*, 2009), using a Waters Alliance 2695 liquid chromatograph (Waters Corporation, Milford, U.S.A.) coupled to a Micromass Quattro Premier XE tandem quadrupole mass spectrometric detector (Micromass Limited, Manchester, U.K.). Chromatographic separation was in reversed phase mode, employing a methanol and water gradient and a phenyl hexyl column (Phenomenex Luna, Torrance, U.S.A.) with dimensions 250 x 4.6 mm (3 μm particle size), thermostatted at 40°C. The gradient started at 35% methanol and increased to 80% in 18 min, followed by column clean-up (95% methanol for 2.4 min) and re-equilibration. The flow rate was 1 mL/min and the total runtime 25 min. This HPLC method is designated Program A in the following discussions. A divert valve was used to direct the column effluent to the detector only between time 9.4 and 20.4 min, the rest being vented to waste. Variable injection volumes were used, with the maximum injection volume being limited to 100 μL . Ionisation was performed in positive ion mode with atmospheric pressure chemical ionisation (APCI). The corona current was 4.4 μA , cone voltage 34 V, and source and desolvation temperatures 150 and 200°C respectively. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, acquiring data for at least two ion transitions per analyte. Compounds

were identified using retention times as well as ion ratios (Table 2).

Qualitative analysis of EMP (HPLC program B)

For qualitative confirmation of EMP and MMP, sample extracts for quantitative analysis were further purified and concentrated as described above. The method for these analyses was identical to the one described above, except that the gradient separation was performed between 25 to 65% methanol and an injection volume of 10 μ L was used. Only EMP and MMP were then eluted in the mass spectral acquisition window to obtain critical separation of the target compounds from interfering peaks. In order to distinguish this procedure from the one described above that is used to separate five MPs, it will be designated HPLC Program B in the following discussion.

LC-UV method for the determination of malic acid

Malic acid was determined by direct injection ion chromatography with UV detection. An Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) fitted with a quaternary pump, autosampler, column oven and UV-visible diode array detector was used. Chromatographic separation was performed in ion exchange mode using a sulphonated (sulphonic acid form) styrene divinyl benzene-based column (Phenomenex, Torrance, U.S.A.) of dimensions 300 x 7.8 mm (8 μ m particle size). The mobile phase was 0.05% (v/v) phosphoric acid in water. The column was thermostatted at 80°C and the flow rate was 0.9 mL/min. The runtime was 20 min and injection volumes of 0.1 μ L were used, while the chromatographic signal was recorded at 210 nm.

Data analysis and statistical methods

Factor analysis and ANOVA were performed utilising Statistica (version 10, StatSoft, Inc., Tulsa, OK, USA) software. Pearson correlation coefficients were used to determine relationships between wine parameters and total methoxypyrazine concentration. Factor analysis was used to further investigate correlation structures between variables and possible latent structures underlying the data. Parallel

analysis was used to determine the number of factors. Main effects ANOVA was used to test for differences in total methoxypyrazine between regions and districts, taking vintage into account. Due to the non-normal nature of the data, log transformations were used.

RESULTS AND DISCUSSION

Performance and validation of the LC-APCI-MS/MS procedure

The LC-MS/MS method for the determination of methoxypyrazines in Sauvignon blanc wine used in this study is based on a procedure that was developed previously for this purpose (Alberts *et al.*, 2009). The method combines the loading capacity inherent to LC with the sensitivity and selectivity of MS/MS in MRM mode, as well as highly concentrated extracts, to achieve very low limits of detection (LODs). The scope of the method reported previously was extended by the addition of MMP and EMP as target analytes (HPLC program A, Chromatographic details).

The system response was linear (r^2 ca. 0.9999) for calibration standards over the range ca. 7 pg to 20 000 pg on-column, corresponding to a maximum sample concentration of 400 ng/L (for a concentration factor of 500 and 100 μ L injection). The limits of detection (LOD) and limits of quantification (LOQ) for the three major MPs (IPMP, IBMP and SBMP) were 0.03 ng/L and 0.10 ng/L respectively. The LOD and LOQ for EMP were 0.10 ng/L and 0.33 ng/L respectively. Although 15 pg (LOD of 0.33 ng/L) MMP could be detected in a standard, the recovery study showed that, in real samples, the signal was obscured by co-eluting matrix components at that concentration. The quantification of MMP therefore only was reliable above levels of 10 ng/L. Compound identification was achieved using retention times as well as the ion ratios for three MRM transitions (Table 2). Three ion transitions were recorded for EMP, namely m/z 138.9 to 123.9, 111.0 and 83.0. For qualitative purposes, a tolerance of 2.5% in retention time and 20% in the ion ratios (relative to calibration standards) was used (O'Keeffe *et al.*, 2003). Fig. 1 shows MRM chromatograms of four ion transitions used to quantify MPs in the extract of a Sauvignon blanc wine.

TABLE 2

MRM transitions and peak identification criteria used for the LC-APCI-MS/MS determination of 3-alkyl-2-methoxypyrazines (ion ratios given in parenthesis).

Compound	Retention time (min)	Target ion (Da)	Product ion (Da)	Transition (ion ratio)	Collision energy (eV)
MMP	10.6	124.9	56.2	2° (2.4)	20
		124.9	97.0	1°	16
EMP	13.8	138.9	111.0	3° (3.4)	16
		138.9	83.0	2° (1.4)	16
		138.9	123.9	1°	20
IPMP	16.7	152.9	122.9	2° (1.1)	26
		152.9	137.9	1°	18
IBMP	18.3	166.9	123.9	2° (1.3)	22
		166.9	124.9	1°	16
SBMP	18.7	166.9	122.9	2° (1.9)	24
		166.9	137.9	1°	18

The accuracy of the method was assessed as the recovery of MPs from samples of Sauvignon blanc wine suitably fortified prior to the sample preparation step. Six replicate sets of recovery measurements were made at three levels of fortification (1, 10 and 100 ng/L). A complete summary of the results of the recovery study, including overall recovery over the range of levels investigated, is presented in Table 3.

The reproducibility of the LC-APCI-MS/MS instrument, expressed as the relative standard deviation (RSD) of the peak area obtained for a standard (IBMP), was better than 2% ($n = 8$). The intra-assay precision, assessed as repeatability of the recovery, was better than 11% (expressed as RSD) at concentrations of 1 ng/L, 10 ng/L and 100 ng/L (IPMP, IBMP and SBMP only). The uncertainty of measurement associated with the procedure was estimated by calculating the range of the 95% confidence interval about the measurements performed in the recovery study (i.e. at levels of 1, 10 and 100 ng/L). Results are reported using the significant figure convention, according to which the result is reported by recording all the certain digits and the first uncertain digit (Skoog *et al.*, 1996). As the limit of quantification of the procedure was determined to be a tenth of 1 ng/L, and the recoveries of the order $85 \pm 5\%$, all results are reported in units of ng/L, using two significant figures.

The accuracy of the LC-APCI-MS/MS method was

confirmed by comparison of quantitative results for the same samples with those obtained by an independent laboratory utilising a validated solid phase micro-extraction GC-MS method. Due to lower of sensitivity of the GC method (LOQ = 5 ng/L), only results for IBMP could be compared. Very good correlation was observed between these two sets of measurements ($r^2 = 0.986$, $n = 6$).

Investigation of the occurrence of MMP and EMP in South African Sauvignon blanc wines

To investigate the occurrence of MMP and EMP, a subset of 10 South African Sauvignon blanc wines was used. EMP was detected and quantified in these samples using the MRM chromatogram of the primary ion transition (m/z 138.9 to 123.9) following analysis with HPLC program A. The secondary and tertiary ion transition peaks (m/z 138.9 to 111.0 and 83.0) were obscured by co-eluting matrix elements so that firm spectral confirmation of the compound could not be obtained according to the criteria given in Table 2. MMP was not detected (< 10 ng/L) in any of the wines analysed. However, since data pertaining to EMP in South African Sauvignon blanc wines are reported here for the first time, firm spectral compound identification was imperative. These extracts were therefore further purified as described under Sample preparation and subsequently

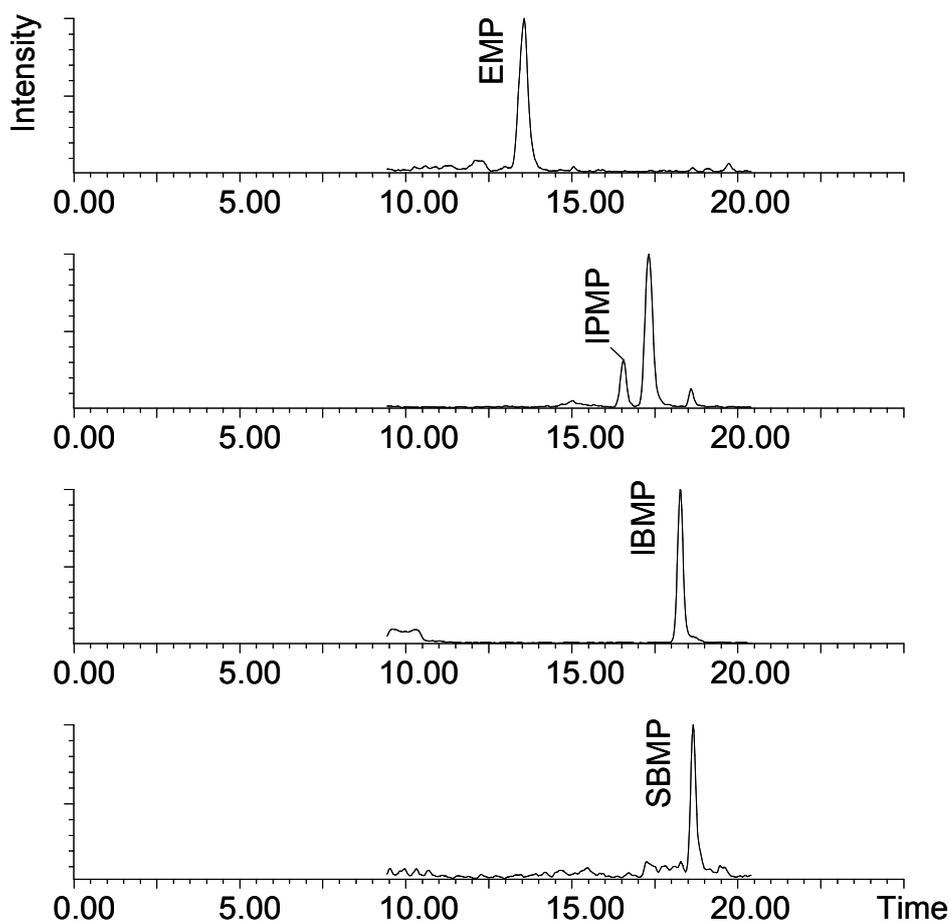


FIGURE 1

MRM chromatograms of four ion transitions used to quantify MPs in extracts of Sauvignon blanc wine. EMP (4.1 $\mu\text{g/L}$), IPMP (0.54 $\mu\text{g/L}$), IBMP (10 $\mu\text{g/L}$), SBMP (0.62 $\mu\text{g/L}$). The injection volume was 100 μL .

re-analysed with a modified HPLC program (Program B, Chromatographic details) to positively identify EMP in these wines. This procedure involved selective back-extraction of the MPs from the concentrated extracts, which served to further enrich the MPs while reducing the interfering sample matrix. The presence of EMP in each sample was inferred unambiguously from the correlation of retention time, as well as two ion ratios for three ion transitions, compared with the reference standard. MRM chromatograms for three ion transitions obtained for the purified extract of a sample of

Sauvignon blanc wine are displayed in Fig. 2.

Augustyn *et al.* (1982) reported the first qualitative data for EMP in Sauvignon blanc wine. Allen and Lacey (1998) tentatively reported EMP concentrations of between 100 and 1 000 ng/L for two red wines. The occurrence of MMP and EMP in wines was subsequently investigated by several further studies utilising headspace solid phase micro-extraction combined with GC and nitrogen-phosphorus selective detection (Sala *et al.*, 2000, 2002) or tandem mass spectrometry (Godelmann *et al.*, 2008), as well as liquid

TABLE 3

Average recovery of MPs at three levels of fortification (1, 10 and 100 ng/L), as well as overall recovery (average recovery over all levels investigated). The relative standard deviation, as a percentage, is given in parenthesis.

Recovery (%)	MMP	EMP	IPMP	IBMP	SBMP
1 ng/L	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6
ave (% RSD)	- ^a	90.8 (28.2)	81.6 (10.3)	95.0 (11.3)	96.8 (5.0)
10 ng/L	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6
ave (% RSD)	65.7 (12.6)	79.6 (5.5)	80.0 (5.5)	87.3 (5.6)	80.4 (4.1)
100 ng/L	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6
ave (% RSD)	62.8 (6.9)	78.1 (4.2)	79.9 (5.1)	87.7 (3.2)	80.6 (3.4)
Overall	<i>n</i> = 12	<i>n</i> = 18	<i>n</i> = 18	<i>n</i> = 18	<i>n</i> = 18
ave (% RSD)	64.3 (10.2)	82.8 (18.6)	80.5 (7.0)	90.0 (8.4)	85.9 (10.1)

^a MMP could not be accurately quantified at this concentration due to matrix interferences

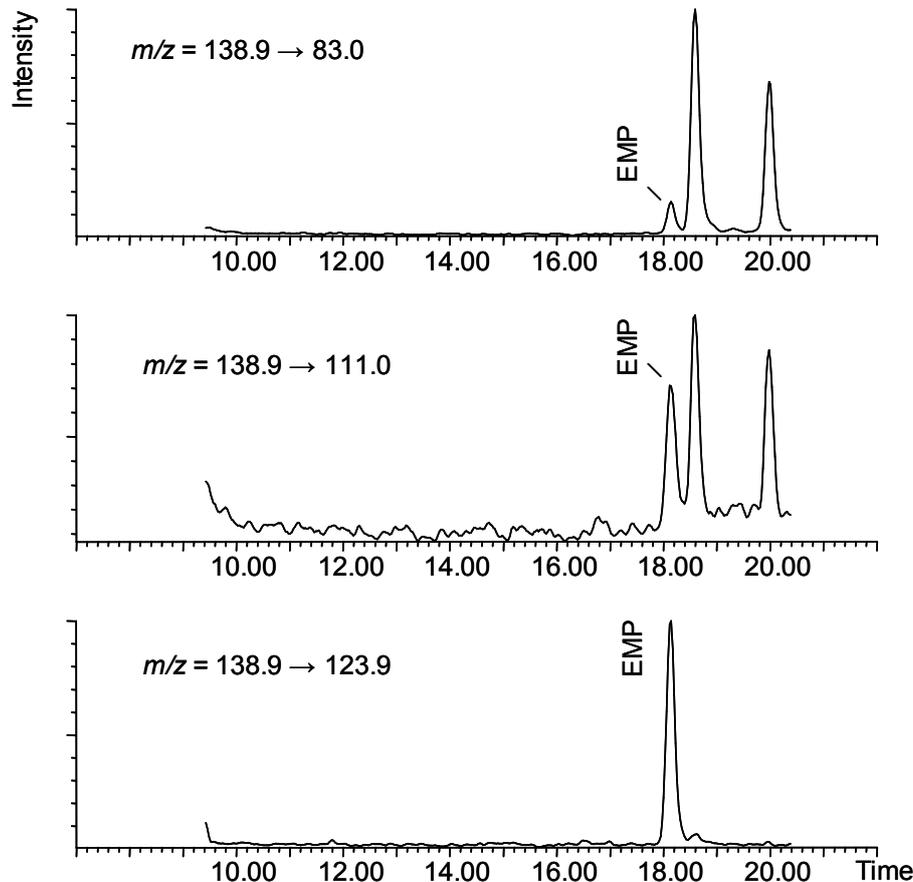


FIGURE 2

MRM chromatograms of three ion transitions obtained for EMP in purified extracts (see Sample preparation) of Sauvignon blanc wine containing 4.9 ng/L EMP utilising LC-APCI-MS/MS (HPLC program B, Chromatographic details).

injection GC-MS (Lacey *et al.*, 1991), but method detection limits (ranging from 0.5 to 40 ng/L) did not allow reliable quantification of this compound. Therefore, apart from a single tentative and unconfirmed report (Allen & Lacey, 1998), the current contribution represents the first report providing accurate quantitative data as well as spectral confirmation of EMP in wine.

The concentration of EMP in the 10 samples analysed varied between 1.1 and 4.9 ng/L, with an average of 2.9 ng/L (RSD of 48%, $n = 10$). At concentrations of this order of magnitude, EMP is not expected to affect the aroma of these wines, considering that its odour threshold in water is 425 ng/L (Allen & Lacey, 1998). As is evident from Fig. 3, the concentrations of EMP in these samples generally co-vary with those of the three other MPs (IPMP, IBMP and SBMP). This observation suggests that the synthesis and degradation of these four MPs may be affected by the same variables (Ryona *et al.*, 2010).

Quantitative survey of the three principal 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wines

Three MPs (IPMP, IBMP and SBMP) have been found to contribute to the characteristic vegetative aroma of Sauvignon blanc wine (Allen *et al.*, 1991; Lacey *et al.*, 1991; Allen & Lacey, 1998; Roujou de Boubee *et al.*, 2000; Sala *et al.*, 2002; Marais, 2004; Marais *et al.*, 2004). Although IBMP is invariably dominant, these compounds have similar odour detection thresholds, and their combined concentrations must be considered in the study of their flavour contribution to these wines (Allen & Lacey, 1998). At a combined concentration of 4 ng/L to 8 ng/L, the herbaceous or vegetative aroma becomes evident in white

wine, while the optimum concentration for Sauvignon blanc wine has been described as 8 ng/L to 15 ng/L. These levels are often difficult to obtain in warmer climates such as South Africa. Sensory perception at concentrations > 30 ng/L are considered overpowering and out of balance (Allen & Lacey, 1998).

In this study, data pertaining to the MP content of South African Sauvignon blanc wines accumulated over four years are reported, comprising a total of 881 samples from all wine-producing regions of South Africa and covering vintages from 1999 to 2011. The IBMP concentration in these samples ranged between 0.40 and 63 ng/L (RSD of 107.4%), with an average of 7.5 ng/L and a median of 4.9 ng/L. Table 4 presents a concise summary of the IBMP concentrations of all the wines, over these vintages, grouped according to geographical origin.

The concentrations of IPMP and SBMP varied from below the LOD (0.03 ng/L) to 7.5 ng/L and 3.3 ng/L respectively, and generally followed similar trends to those observed for IBMP. The ratios of IPMP to IBMP and SBMP to IBMP were approximately 10% in each case, and these displayed less variation than the overall IBMP concentration. These data follow similar trends compared to our previous study for a smaller set of wines (Alberts *et al.*, 2009). However, the addition of 304 samples spanning a wider range of vintages and extending the representation of geographical origins has improved the statistical representation of the South African Sauvignon blanc industry to increase the validity of conclusions drawn from the data. Furthermore, the malic acid content was determined for a subset of samples, which were used together with general wine parameters to investigate the correlation of these parameters with methoxypyrazine concentrations.

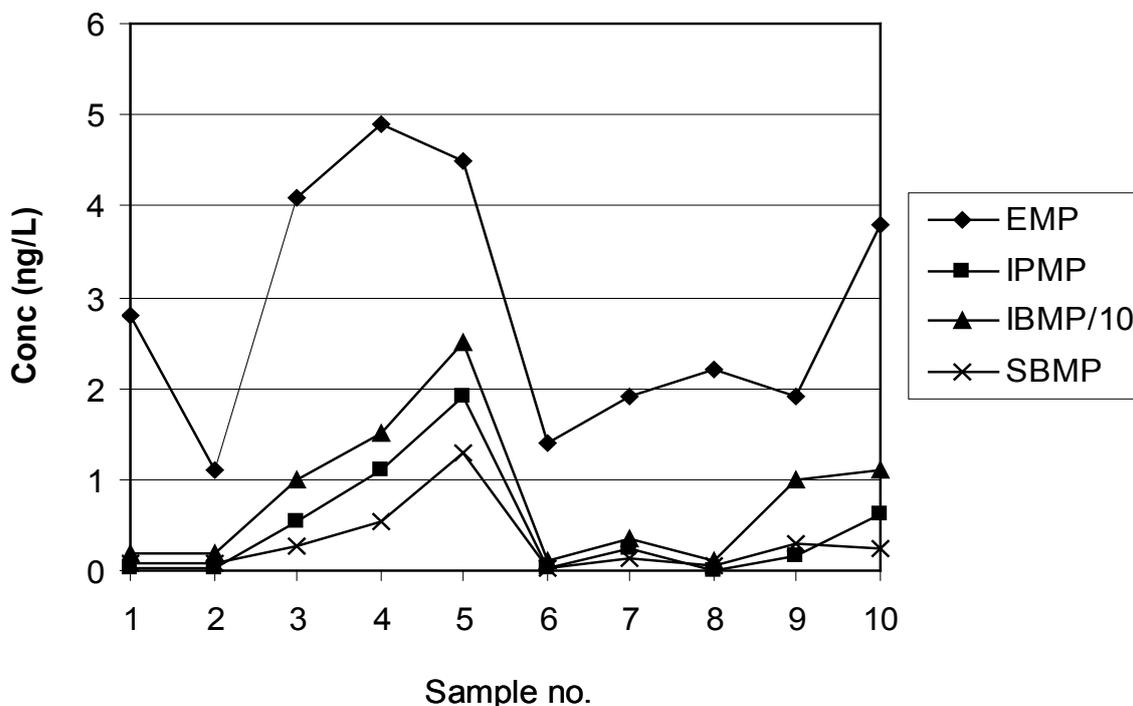


FIGURE 3

Concentrations of four MPs in 10 samples of South African Sauvignon blanc wine. IBMP attenuated (by a factor of 10) to enhance the readability of the figure.

TABLE 4
IBMP levels (ng/L) in South African Sauvignon blanc wines from different geographical origins over vintages 1999 to 2011.

Region / district / ward	<i>n</i>	Minimum	Maximum	Average	% RSD
Breede River Valley	152	0.60	53	5.7	122.4
- Breedekloof ^a	20	0.89	26	8.0	81.6
- Robertson ^a	81	0.63	53	6.0	138.5
- Worcester ^a	42	0.60	17	4.7	83.2
Coastal Region	402	0.40	63	8.5	100.1
- Cape Point ^a	4	11	62	25	97.2
- Constantia ^b	25	6.1	29	12	46.1
- Darling ^a	18	4.6	39	14	53.7
- Paarl ^a	61	0.52	24	4.3	123.8
- Tygerberg ^a	33	0.74	38	11	74.6
- Stellenbosch ^a	162	0.76	63	7.8	113.9
- Swartland ^a	5	1.3	5.5	3.4	44.4
- Tulbagh ^a	3	1.9	2.2	2.0	7.5
Olifants River	6	3.5	45	17	104.6
- Lutzville Valley ^a	4	4.6	45	22	90.0
Cape Agulhas ^a	18	4.2	42	17	65.8
Overberg ^a	22	1.3	26	9.1	85.7
Walker bay ^a	18	1.2	25	7.2	85.4
Cederberg ^b	5	3.3	16	10	55.2
Prince Albert Valley ^b	3	0.81	4.2	2.0	97.8
Little Karoo	3	1.4	6.5	3.2	87.8
Western Cape ^c	250	0.48	44	6.1	112.0

^a District

^b Ward

^c All wine-producing areas of the Western Cape

Two outstanding features in these data are the large variance in (RSD of 108.5%) and asymmetric distribution of (skewness of distribution around mean = 2.8) the total MP concentration of these samples (where total MP concentration refers to the sum of IBMP, IPMP and SBMP). In Fig. 4, the predicted flavour contribution of the total MPs (based on Allen & Lacey, 1998) to the analysed South African wines is given as a function of percentage of the total number of samples. It should be noted that, due to synergistic interactions between various aroma compounds, sensory properties and consumer preference of Sauvignon blanc wines are difficult to predict with great precision (Escudero *et al.*, 2007; King *et al.*, 2011), and the flavour descriptions used in the current context refer to the probable effect that these compounds may have on the aroma of the wines. The predicted flavour descriptions range from below recognition through to overpowering and out of balance, with 18% of the samples falling in the narrow concentration window where their contribution may be regarded as positively favourable to the vegetative Sauvignon blanc wine aroma. The majority of the samples were at the lower end of the scale, with 38% of the wines classified as below recognition, while in 30% of the cases the green pepper aroma should be recognisable based on the MP concentrations.

Statistical investigation of the relationship between the viticultural parameters and 3-alkyl-2-methoxyypyrazine content of South African Sauvignon blanc wines

In general, the timing of the harvest is a very important viticultural parameter, as wine quality is limited by the properties of the fruit used in the vinification process. During the final stages of maturation, grape sugars and acids undergo important quantitative changes (see further), and the optimal harvest date ultimately depends on the intended style of the final product. At the same time, these parameters are also affected by climatic and viticultural factors (Jackson, 2000). Therefore, wine parameters such as acidity, pH and alcohol content may reveal information related to factors such as the maturity of the grapes at the time of harvesting, as well as climatic and viticultural conditions. Since wine MP concentrations are also strongly and systematically influenced by these viticultural factors, their correlation with wine parameters such as TA, VA, malic acid, pH and Alc were investigated for a subset ($n = 158$) of wines using two-dimensional and multivariate data analysis techniques. In addition, differences between vintages and regions of origin were elucidated (for the entire dataset, $n = 881$) using methoxyypyrazine data for the three principal congeners in order to assess the effects of annual climatological variations and geographic differences.

Apart from the change in sugar content, the reduction

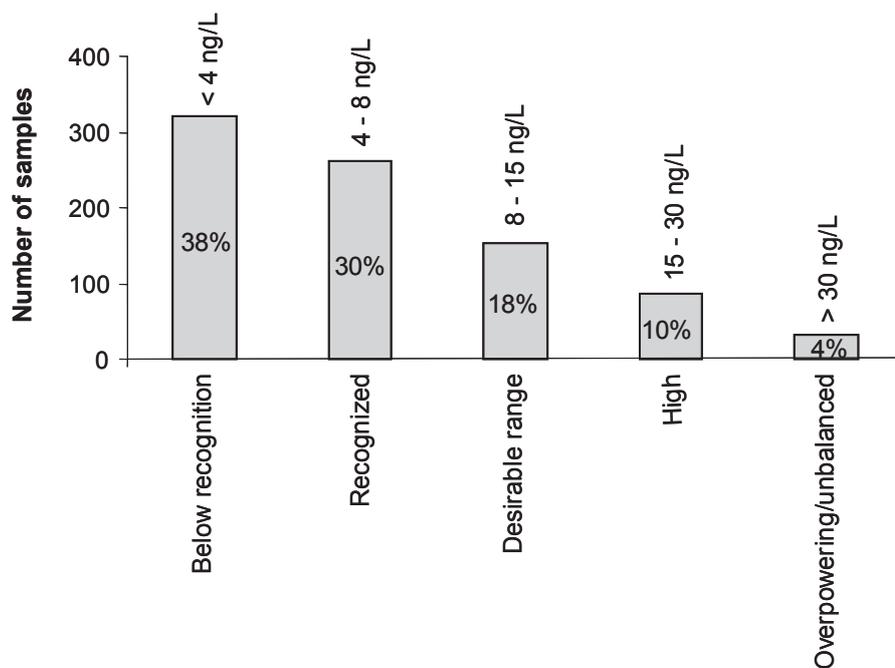


FIGURE 4

The predicted aroma contribution (based on Allen and Lacey, 1998) of total MPs as a function of frequency in 881 South African Sauvignon blanc wines.

of acidity is quantitatively the largest chemical change to occur in grapes during ripening. Tartaric and malic acids are quantitatively the most abundant grape acids, accounting for approximately 70 to 90% of the berry acid content. The levels of these acids are determined by complex factors, but certain trends are generally observed. While the tartaric acid content remains relatively stable after véraison, the malic acid content of grapes declines drastically during maturation. The rate of decline in berry malic acid is cultivar dependent and is also strongly influenced by environmental factors. It is generally accepted that grapes grown in hot climates often metabolise most of their malic acid before harvest, whereas grapes grown in cooler climates retain most of their malic acid into maturity (Jackson, 2000). As a result, a relatively high wine malic acid concentration may indicate that the grapes were harvested earlier or were grown in cool conditions, both of which are also factors that are associated with higher levels of wine MPs (Hashizume & Samuta, 1999; Marais *et al.*, 1999; Roujou de Boubee *et al.*, 2000; Marais, 2004; Sala *et al.*, 2004; Ryona *et al.*, 2010). This hypothesis is supported by a study that reported that the breakdown of malic acid and IBMP in grapes during ripening is highly correlated (r^2 of 0.90 to 0.99), irrespective of soil type, grape variety or vintage (Roujou de Boubee *et al.*, 2000). The malic acid content of a wine may therefore reveal information regarding cultivation microclimate and maturity of the grapes and, by extension, the methoxypyrazine potential of the product.

In addition, it has also been reported that excessive vigour (the relationship between foliage and fruit) is consistently correlated with reduced acidity and high wine pH (Jackson, 2000). Vigour, in turn, is also correlated with higher levels of wine MPs, mainly as a result of the shading of bunches during ripening (Marais *et al.*, 1999). Low wine total acidity and high pH, when resulting from

increased vigour, may therefore be correlated with higher levels of wine MPs. These phenomena (early harvesting and vigour) are generally associated with higher levels of wine MPs, but they have opposing effects on wine pH and acidity. However, since the level of tartaric acid is relatively stable after véraison, the ratio of tartaric to malic acid may be more significant in predicting the levels of wine MPs.

These hypotheses were investigated to determine whether the MP content of Sauvignon blanc wines was correlated with wine parameters such as malic acid content, acidity, pH and alcohol content. Note that reducing sugars are not expected to provide useful information in these analyses, since the practice of sweetening may alter this parameter independent of production conditions.

Two-dimensional plots of wine parameters such as Alc, pH, TA, VA and malic acid vs. the total wine MP concentration for the subset of 158 samples produced considerable scattering of data-points. Alc (Pearson correlation coefficient, $r = 0.04$, $p = 0.65$) and pH ($r = 0.05$, $p = 0.52$) did not show significant co-variance with total MP content, whereas statistically significant (although weak) positive correlation was observed between the variables TA ($r = 0.29$, $p < 0.01$), VA ($r = 0.30$, $p < 0.01$) and malic acid ($r = 0.34$, $p < 0.01$) and total wine MP concentration. Samples with high levels of TA, VA and malic acid therefore generally also contained higher levels of MPs, while Alc and pH displayed little systematic variance as a function of MP concentration. Despite these observations, the low correlation coefficients indicate significant scatter in the data, implying that additional factors also affect these variables.

Nature is multivariate in the sense that particular phenomena, such as wine methoxypyrazine content, usually depend on several factors, such as time of harvest, vigour, solar radiation, canopy microclimate, mesoclimate, as well

as oenological and viticultural practices. Each of these production factors also affects the general wine parameters, such as Alc, pH, TA, VA and malic acid. The multivariate data analysis technique factor analysis was therefore used to determine whether intrinsic correlations exist between wine MP concentrations and these parameters, since this method is suitable for reducing the dimensionality of data by extracting interpretable factors. By reducing multivariate data to a few sets of factors of physical significance, the visual representation of these relationships is enhanced and meaningful interpretation of the data is facilitated. In this way the underlying correlations and qualitative features in the data can be identified.

In the first instance, parallel analysis was performed on the data subset ($n = 158$), for which malic acid was determined to give an indication of the number of factors underlying the original data. Two factors were identified that explained 60.5% of the total variance. The loadings plot for these two factors is displayed in Fig. 5. It is evident from this analysis that the concentrations of the three principal wine MPs are highly correlated, and mainly are responsible for the variation described by Factor 1 (F1). This implies that, for wines rich in IBMP, the concentrations of IPMP and SBMP are invariably correspondingly high, and *vice versa*. This information suggests that their occurrence in wine is regulated by the same vinivicultural variables.

Alc and pH displayed a negative correlation. These variables showed little co-variance as a function of MP concentration (F1), but rather are associated with the variance described by Factor 2 (F2). Malic acid, TA and VA are to a similar extent also positively correlated with MP concentration, and therefore with the variance described by F1. Of all the wine parameters, TA shows the highest correlation with wine MPs - a phenomenon that may be explained by noting that relatively early harvesting and cool growing conditions result in higher grape acidity and also are favourable for producing wines containing high levels of MPs. On the other hand, relatively late harvesting and warm growing conditions may produce lower grape acidity and also lower wine MPs. The correlation of malic acid with wine MPs supports this observation, as malic acid retention may be ascribed to early harvesting or cool growing conditions - factors that are conducive to high wine MPs. The correlation of malic acid levels with MPs observed here is, however, not as clearly defined as the association reported by Roujou de Boubee *et al.* (2000).

Acetic acid is the main volatile acid in wine and, in the absence of spoilage, it occurs principally as a by-product of yeast and bacterial metabolism (Jackson, 2000). It has been reported that different yeast strains have an effect on the yield of wine MPs from the juice (Marais, 2004; Pickering *et al.*, 2008). The correlation of VA with MP levels therefore possibly may be ascribed to the effect of different yeast strains on the production of acetic acid and MPs in the wine. This assumption is also supported by the correlation of VA and Alc, since it is well known that different yeast strains cause differences in the production of wine ethanol.

The positive correlation of malic acid content with pH indicates cool growing conditions, conducive to the retention of grape malic acid into maturity, while simultaneously also

leading to low TA and high wine pH. The same phenomenon also explains the negative correlation of malic acid with Alc, as cool growing conditions, which favour malic acid retention, also produce lower levels of sugars and less alcoholic wines. The fact that pH is not correlated at all with F1 might be due to the opposing effects of vigour (lower acidity) and early harvesting (higher acidity) on wine pH, while both these factors are conducive to higher wine MPs. The negative correlation of pH and Alc confirms the validity of these assumptions, as cool growing conditions and vigour lead to the production of fewer acids (higher wine pH) and lower sugars (lower wine alcohol) at the same time. On the other hand, riper grapes contain higher levels of sugars and lower levels of acids, yielding higher pH and more alcoholic wines. The fact that this phenomenon (wines with high pH and high alcohol content) is not observed in this analysis demonstrates the involvement of cool growing conditions and vigour in the production of these wines.

Surprisingly, a positive correlation between Alc and TA was found for the analysed wines. It would be expected that higher acidity, resulting from early harvesting and cool growing conditions, would simultaneously also lead to the production of lower sugars and less alcoholic wines.

From these data it would appear that climatic conditions, grape ripeness and vigour all play a determinative role in the MP content of Sauvignon blanc wines. Furthermore, while general wine parameters such as TA, VA and malic acid concentration show some correlation with MP levels, they are not absolute indicators of the MP content of Sauvignon blanc wines, due to the complex interplay between variables affecting MP concentration in the final wine.

Variations in 3-alkyl-2-methoxypyrazine content of South African Sauvignon blanc wines as a function of geographical origin

In wine production, certain factors that have an effect on the product are related to its geographical origin. Aspects such as soil influences (for example geological origin, texture, structure, drainage and water availability, depth, nutrient content and pH, organic content and colour), topographic influences (for example solar exposure, wind direction, frost and winter protection, altitude and drainage) and atmospheric influences (such as temperature, solar radiation, wind and water availability) have an impact on the final product to varying extents. This point is amply illustrated by noting that regions affected by continental influences may have more than twice the average day-night temperature variation compared to an equivalent maritime region. In addition, seasonal climatic variations may manifest changes in the product as a function of vintage (Jackson, 2000). In grapes, the concentrations of MPs are strongly influenced by *terroir* (soil, climatic and vinivicultural influences) (Lacey *et al.*, 1991; Allen & Lacey, 1998; Hashizume & Samuta, 1999; Marais *et al.*, 1999; Roujou de Boubee *et al.*, 2000; Marais *et al.*, 2004; Sala *et al.*, 2004, 2005; Maggu *et al.*, 2007). The systematic effect of geographical origin and vintage on the total MP concentration of South African Sauvignon blanc wines therefore was investigated using main effects ANOVA.

Firstly, classification according to region of origin was

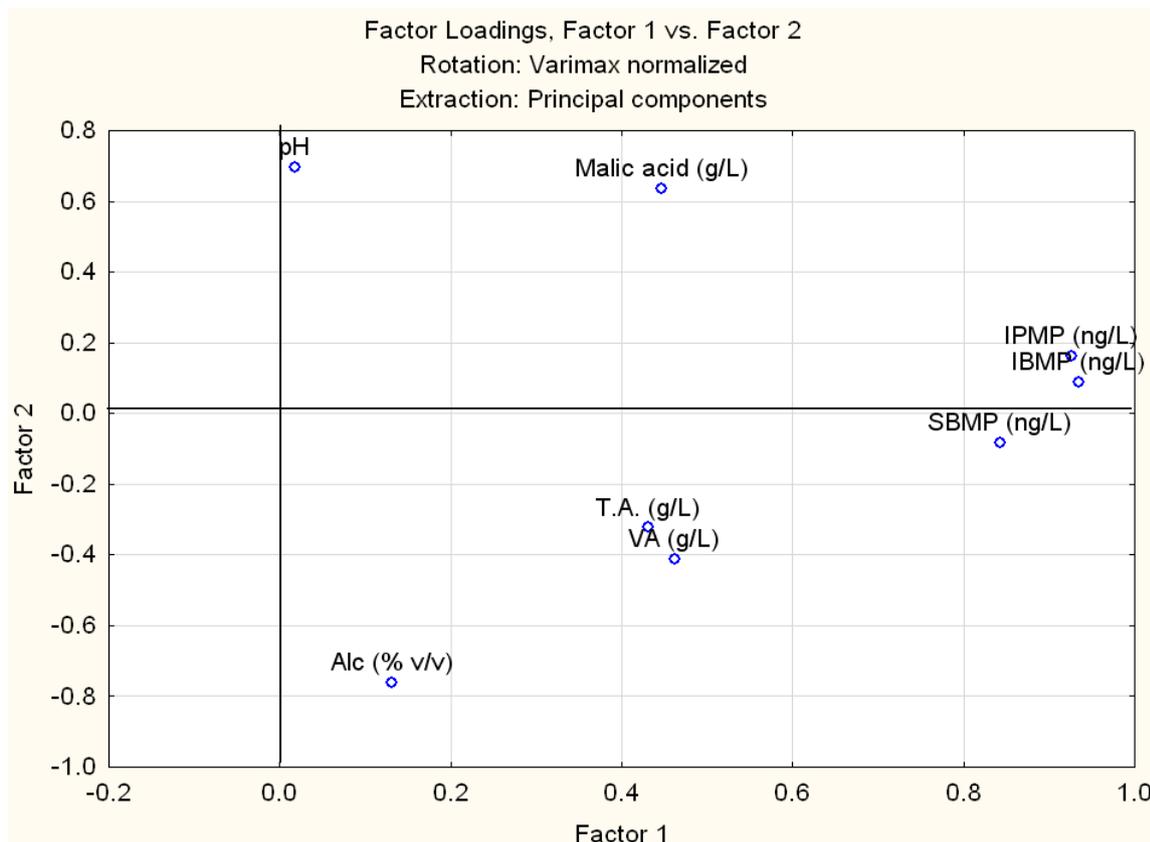


FIGURE 5

Loadings plot for factor analysis of two principal factors explaining 38.2 and 22.3% of the total variance respectively, obtained for the data subset of 158 wines.

investigated. In the South African wine of origin scheme, wines may be certified for region of origin by classification into regions, districts or wards (see Table 4). Of these categories, region is the broadest category of classification. Samples from all vintages in the dataset were therefore grouped according to region, where appropriate, while all non-specific origins (such as Western Cape) and minor regions (for which insufficient data were available) were removed. A data subset of 554 samples was obtained, of which 145 samples originated from the Breede River Valley and 385 samples from the Coastal Region. This subset was analysed by ANOVA to determine whether these origins can be distinguished based on the total MP concentration of these wines. The mean of the Breede River Valley group (6.15 ng/L) was lower than that of Coastal Region (9.17 ng/L, $p < 0.01$). This result possibly indicates slightly warmer growing conditions prevalent in the Breede River Valley compared to the Coastal Region. Despite the relatively wide limits for the 95% confidence interval, which indicate significant variation in MP levels, statistically significant differences in mean values were found between these two regions.

ANOVA was subsequently used to elucidate grouping of samples from different districts located in the greater Breede River Valley and Coastal Region respectively, using total MP data. By performing main effects ANOVA using wine samples for those districts that are represented by a statistically significant number of samples and over vintages 2003 to 2011, significant differences between these districts were elucidated, and in this way the effect of variation

over vintages was taken into account. Fig. 6A illustrates that there are clear differences between some districts in terms of total wine MP content. For example, Overberg and Tygerberg were clearly distinct from Paarl and Robertson, based on higher levels of MPs. Overberg and Tygerberg are two districts located in relative close proximity to the Atlantic Ocean, and this result possibly indicates favourable viticultural conditions for Sauvignon blanc production in these locations. On the other hand, this analysis indicates that Paarl and Robertson represent districts that are characterised by warmer climates and generally produce wines that contain lower levels of MPs. Since this analysis represents a large number of samples ($n = 382$) and is independent of vintage, it is clear that statistically significant differences ($p < 0.01$) exist in wine MP levels between different districts. This shows that certain districts inherently lend themselves to the production of Sauvignon blanc wines of higher MP content. This observation is in agreement with the work of Marais *et al.* (1999), who also observed the suitability of distinct localities for Sauvignon blanc production.

Note that discrepancies in the number of samples between the districts, as well as variance in the data for each district, affect the results reported in Fig. 6A. For example, Stellenbosch represents the largest number of samples ($n = 158$), while Overberg ($n = 20$) is represented by much lower numbers, which explains the larger 95% confidence interval for the latter. As before, these significant differences cannot be used to classify wines absolutely according to districts of origin, due to the relatively large variation in each group.

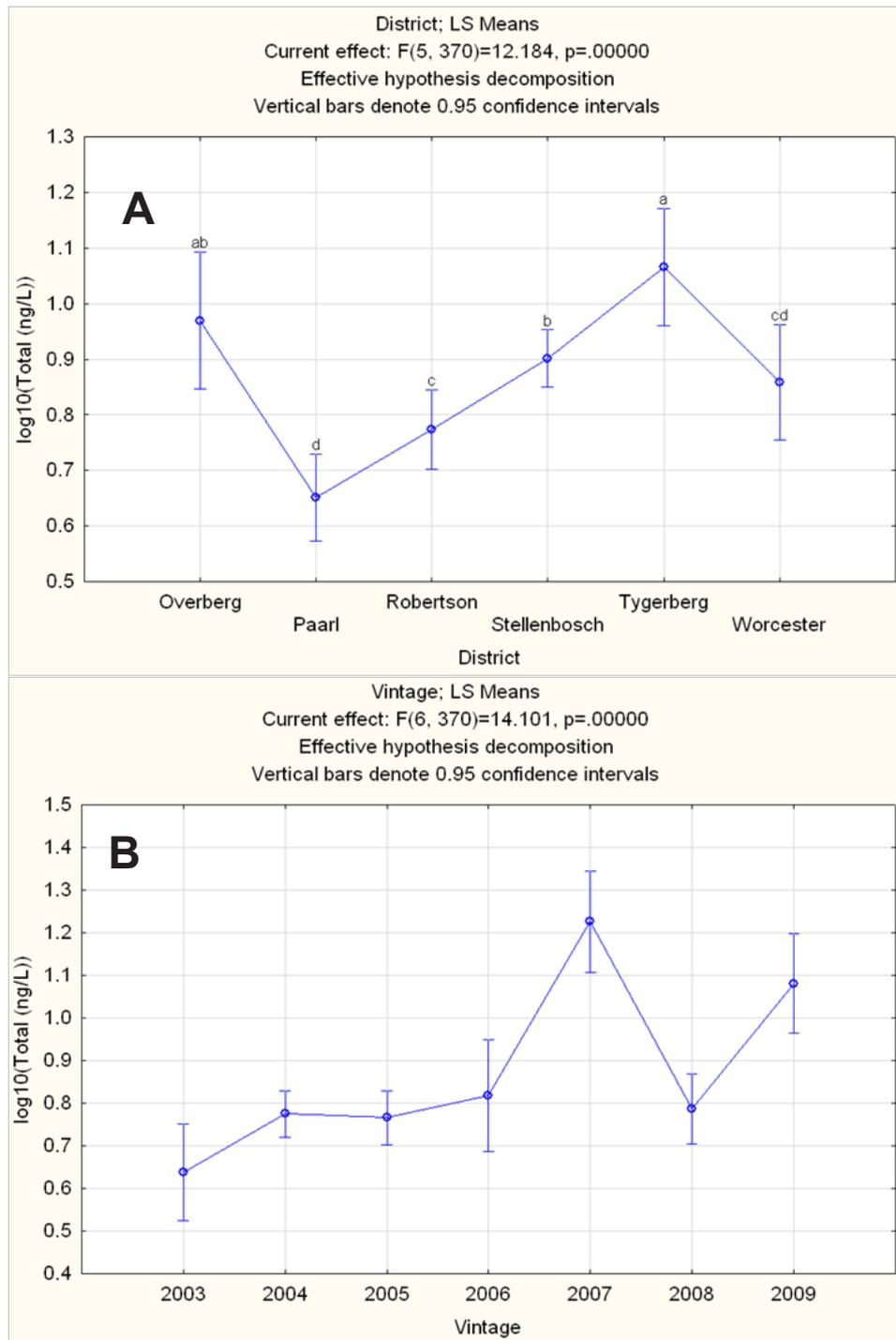


FIGURE 6

ANOVA results for determining significant differences between (A) districts and (B) vintages between 2003 and 2009, based on total wine methoxyppyrazine concentration.

Variation in 3-alkyl-2-methoxyppyrazine content of South African Sauvignon blanc wines as a function of vintage

The effect of climatic variations on total wine MP concentration was evaluated by comparing mean MP levels in the total dataset between vintages. Samples from 2003 to 2009 ($n = 382$) were compared, controlling for district in a main effects ANOVA. From the information in Fig. 6B, it is clear that wines with relatively high levels of MPs were produced in 2007 and 2009. Since the data are independent of geographical origin, this result suggests that favourable

climatological conditions (such as lower temperatures, higher rainfall and/or lower solar radiation) were prevalent in the 2007 and 2009 seasons compared to other vintages. It should be noted that ageing had no measurable effect on methoxyppyrazine concentrations for bottled wines, as confirmed by the analysis of the same wines in consecutive years.

Finally, it should be noted that the commercial products analysed in this study, although labelled as single varietal wines, may contain between 15 and 25% of other varieties

(depending on vintage). In addition, wines certified for vintage may contain up to 15% of wine from the preceding or subsequent years (Liquor Products Act, 1989). This additional variability can certainly contribute to the observed variance in MP concentrations, particularly considering that the grapevine synthesis of MPs is highly dependent on genotype (Koch *et al.*, 2010), and because of climatic variations between vintages (Lacey *et al.*, 1991; Falcão *et al.*, 2007).

CONCLUSION

In this study, an LC-APCI-MS/MS method was applied to generate the first extensive database of the levels of 3-alkyl-2-methoxy-pyrazines in 881 samples of South African Sauvignon blanc wines spanning 13 vintages and all the major wine-producing regions of the country. The total MP concentration (sum of IPMP, IBMP and SBMP) in these samples ranged from 0.52 to 72 ng/L, suggesting that the typical green pepper aroma of these products may range from below recognition through to overpowering and out of balance. Their combined concentrations exceeded the recognition threshold for the vegetative aroma in more than 60% of the samples analysed, demonstrating the extent of involvement of these compounds in the typical aroma of the cultivar.

The first confirmation of 3-ethyl-2-methoxy-pyrazine in Sauvignon blanc wines is reported, and this compound was accurately quantified in a subset of 10 samples using modified extraction and analysis procedures. The concentrations of 3-ethyl-2-methoxy-pyrazine were two orders of magnitude below its odour detection threshold, which makes it highly unlikely that this methoxy-pyrazine contributes significantly to the aroma of these wines.

Statistical analysis of the total MP content data using factor analysis shows that the concentrations of IPMP, IBMP and SBMP are highly correlated, suggesting that their occurrence in Sauvignon blanc wines is regulated by the same factors. In addition, factor analysis revealed that methoxy-pyrazines are correlated with total acidity, volatile acids and malic acid. Higher acidity may result from earlier harvesting, while higher levels of malic acid in particular indicate cooler climates or early harvesting, vinicultural conditions that are also favourable for the production of wines containing higher levels of MPs. The observed correlation between MP levels and volatile acids could possibly be ascribed to the effect of different yeast strains on the yield of MPs from the juice. Statistical analysis using ANOVA showed significant differences between wines from different geographical origins (independent of vintage) and vintages (independent of geographical origin). The Overberg and Tygerberg districts appear to be particularly suited for the production of varietal-typical vegetative Sauvignon blanc wines, as they displayed higher average total MP concentrations, while the 2007 and 2009 vintages also produced wines that contained higher average levels of MPs across all regions. The data presented in this paper sheds much-needed light on the MP content of representative South African Sauvignon blanc wines and the factors affecting the levels of these influential aroma compounds.

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