Changes of 3-Alkyl-2-methoxypyrazines in Developing Cabernet Sauvignon (*Vitis vinifera*) and Zuoshanyi (*Vitis amurensis* Rupr.) Grapes from North China

B. Zhu[1†], Y. Wu[1,2†], X. Xu[1], Q. Pan[1], C. Duan[1]*

(1) Center for Viticulture and Enology, College of Food Science & Nutritional Engineering, China Agricultural University, Beijing 100083, China
(2) COFCO Wine Industry Co. Ltd., Beijing 100005, China

Submitted for publication: January 2012
Accepted for publication: February 2012

Key words: IBMP, IPMP, Cabernet Sauvignon, Zuoshanyi, *Vitis amurensis* Rupr.

Changes in 3-alkyl-2-methoxypyrazines (MPs) in Cabernet Sauvignon (*Vitis vinifera*) and Zuoshanyi (*Vitis amurensis* Rupr.) grapes from different areas of North China were monitored throughout berry development in 2010. One kind of MP, 3-isobutyl-2-methoxypyrazine (IBMP), was detected in Cabernet Sauvignon grapes, and its content in grapes from Gaotai County was much higher than in those from the other two producing areas at the stages of véraison and maturity. Zuoshanyi grapes contained much more IBMP than Cabernet Sauvignon grapes, and an additional kind of MP, 3-isopropyl-2-methoxypyrazine (IPMP), was also detected in developing Zuoshanyi grapes. Abundant MPs might explain the stronger 'green' and 'herbaceous' characters in Zuoshanyi grapes. MPs accumulated in the early developmental stage in both Zuoshanyi and Cabernet Sauvignon grapes and rapidly decreased in the later stages. Delays in the time of harvest for Cabernet Sauvignon in Gaotai County and Zuoshanyi in Beijing are suggested in order to reduce MPs levels and the herbaceous character of these grapes.

INTRODUCTION

Grape-derived 3-alkyl-2-methoxypyrazines (MPs), particularly 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (*s*-BMP), have been reported to be important components affecting the flavor of some wines, exhibiting a scent of vegetative, herbaceous and bell pepper character due to their low sensory thresholds (ng/L level) (Lacey *et al*., 1991; Sala *et al*., 2005; Ryona *et al*., 2008; Dunlevy *et al*., 2009; Ryona *et al*., 2010). Although these MPs can contribute positively to wine quality in some *Vitis vinifera* varieties (Parr *et al*., 2007), an excessive concentration of MPs will mask "fruity and floral" aromas and reduce consumer acceptance (Ryona *et al*., 2008). Generally, IBMP is the most abundant MP compound and has a stronger impact on the final wines than the others (Lacey *et al*., 1991; Koch *et al*., 2010).

MPs have been considered to be the varietal aroma components of Cabernet Sauvignon, Cabernet Franc, Sauvignon blanc, Merlot and Carmenere (Lacey *et al*., 1991; Sala *et al*., 2002; Chapman *et al*., 2004; Sala *et al*., 2005; Belancic & Agosin, 2007; Falcão *et al*., 2007; Ryona *et al*., 2008, 2010), although they are also found in other varieties of *Vitis vinifera*, such as Chardonnay, Pinot Noir, Riesling and Semillon (Hashizume & Samuta, 1999; Ryona *et al*., 2010). In *Vitis vinifera* grapes, MPs accumulate at pre-véraison and then decrease markedly until maturity (Ryona *et al*., 2008; Dunlevy *et al*., 2010). The contents of MPs in grapes are affected by cluster light exposure, yield, irrigation condition, vine training, plantation density, seasonal temperature, vineyard altitude and soil (Hashizume & Samuta, 1999; De Boubée *et al*., 2000; Chapman *et al*., 2004; Sala *et al*., 2004, 2005; Belancic & Agosin, 2007; Falcão *et al*., 2007; Ryona *et al*., 2008; Dunlevy *et al*., 2009). In addition, IPMP content was reported to be higher in must and wines contaminated by the multicoloured Asian ladybug (Pickering *et al*., 2004). For viticulturists, it is necessary to understand the factors affecting the formation and disappearance of MPs in grapes and to control their levels at those having a positive effect through vineyard practices.

Over the past 30 years, increasing numbers of vineyards have been located in North China, from the east coast (Changli County) to the Huaizhuo Basin (Huailai County), and in the northwest inland (Gaotai County). The growing environment for grapes (climate, soil, etc.) differs greatly among these producing areas and it is hypothesised that the development of MPs in Cabernet Sauvignon grapes might

**Corresponding author: chqduan@yahoo.com.cn [Tel & Fax: +86-10-62737136]. †These authors contributed equally to this work*
Acknowledgements: This work was supported financially by the China Agriculture Research System (No. CARS-30).

vary in these areas of North China. So far, no report has been found on this topic. Zuoshanyi (*Vitis amurensis* Rupr.) is a special grape variety for winemaking that is native to the northeast of China. This cultivar is famous for its outstanding cold resistance, and its wine is characterised by high acidity and a dark-red colour (He & Chao, 1982; Zhao *et al*., 2010). However, Zuoshanyi grapes and wines possess a strong smell of vegetal or green, and this character severely reduces the quality of the wine. This study aims to investigate the differences in the developmental trends of MPs in Cabernet Sauvignon grapes from the Gaotai, Huailai and Changli areas, as well as in Zuoshanyi grapes from Beijing, in order to provide basic data for wine quality improvement by controlling the content of MPs in grape materials through vineyard management and vinification techniques.

MATERIALS AND METHODS

Materials

All grape samples were collected at fortnightly intervals from two weeks after flowering until harvest in 2010, following the method described by Jin *et al*. (2009). Cabernet Sauvignon (*Vitis vinifera* L.) grapes were sampled from three producing areas: Gaotai County in Gansu Province (39.14°N, 99.84°E), and Huailai County (40.40°N, 115.54°E) and Changli County (39.72°N, 119.15°E) in Heibei Province. Zuoshanyi (*Vitis amurensis* Rupr.) grapes were sampled from Beijing (40.10°N, 116.20°E). The average daily temperatures of these producing areas in 2010 are shown in Fig. 1. Gaotai County was characterised by a relatively lower temperature than Huailai County and Changli County.

Three 100-berry samples were collected randomly and the sampling was performed at 09:00 to 10:00. These samples were then rapidly frozen in liquid nitrogen and kept at -80°C until analysis.

Chemicals

Tartaric acid (≥ 98%, capillary GC) was purchased from Sigma-Aldrich (China sector), and NaOH and NaCl (analytical grade) were purchased from Beijing Chemical Works. The reagents of IBMP (99.5% pure, Dr Ehrenstorfer GmbH, Augsburg, Germany) and IPMP (97.0% pure, Aldrich, Milwaukee, WI) were used as reference standards, and 4-methyl-2-pentanol (98.0% pure, Aldrich, Milwaukee, WI) was used as internal standard.

Sample preparation

One hundred grams of fruit from each sample was ground and blended with 1 g PVPP. After being macerated at 4°C for 140 min, the flesh was immediately centrifuged at 2000 × *g* and 4°C for 10 min, and clear juice was obtained. Total soluble solids (as ºBrix) and the pH of each juice sample were measured to describe the stage of grape maturity. The results are showed in Table 1. Five millilitres of the juice, 10 µl 4-methyl-2-pentanol (1.039 mg/ml water, internal standard) and 1 g of NaCl were blended in a 15 mL sample vial tightly capped with a PTFE-silicon septum and containing a magnetic stirrer. Afterwards, the vial containing the sample was placed on a heating platform for agitation (hot/stirrer plate) at 40°C for 30 min. The pre-treated SPME fibre (50/30 µm DVB/Carboxen/PDMS, Supelco, Bellefonte, Pa., USA) was then inserted into the headspace, extracting for 30 min with continuous heating and agitation. The fibre

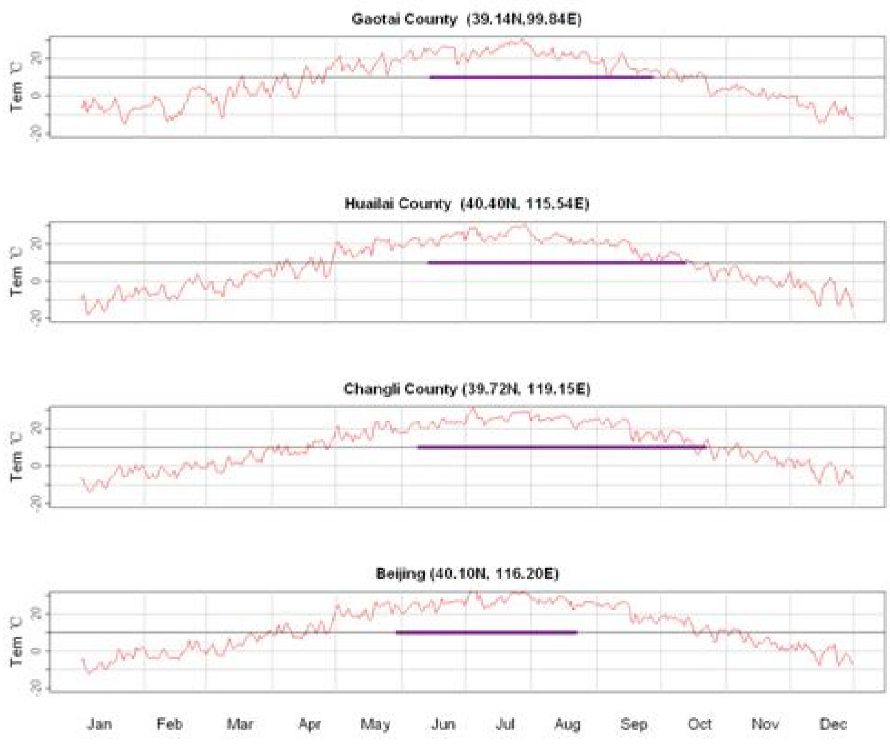

FIGURE 1
Changes in average daily temperature of the Gaotai, Huailai, Changli and Beijing areas in 2010. In each figure, the bold purple line covers the period from flowering to harvest of the grapes in the corresponding producing area.

TABLE 1
Changes of total soluble solid (TTS; as ºBrix) and pH in developing Cabernet Sauvignon (CS) grapes from the Gaotai (G), Huailai (H) and Changli (C) areas, as well as Zuoshanyi grapes from Beijing.

Samples	Weeks after flowering																			
	2		4		6		8		10		12		14		15		16		17	
	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH
CS-G	4.1	2.7	3.9	2.6	4.1	2.7	7.4	2.7	12.9	2.9	13.5	3.0	17.8	3.2	18.9	3.4	-	-	-	-
CS-H	3.0	2.7	3.7	2.7	4.0	2.9	12.0	3.0	15.2	3.4	16.7	3.5	18.4	3.7	-	-	20.1	3.8	-	-
CS-C	4.1	2.7	3.8	2.8	5.4	2.8	11.5	2.9	14.6	3.1	15.8	3.3	17.6	3.5	-	-	18.9	3.6	20.8	3.7
Zuoshanyi	3.3	2.8	3.4	2.9	3.2	2.9	3.1	2.9	9.0	2.8	15.1	3.1	16.8	3.3	-	-	-	-	-	-

was instantly desorbed in the GC injector for 8 min. Three independent extractions were done for each sample.

GC-MS conditions

An Agilent 6890 GC equipped with an Agilent 5975 MS and fitted with a 60 m × 0.25 mm id HP-INNOWAX capillary column with 0.25 μm film thickness (J&W Scientific, Folsom, Calif., USA) was employed to separate and identify the aromatic volatiles. The conditions of GC-MS were as reported previously (Wu *et al*., 2009). IBMP, IPMP and 4-methyl-2-pentanol were measured using the selected ion monitoring (SIM) mode, and 124, 137 and 45 were used for quantification respectively. Each independent extraction was carried out in duplicate.

Quantification

A model solution was prepared in distilled water containing 7 g/L tartaric acid, 200 g/L glucose and some representative volatile components, such as hexanal, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*Z*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, 1-hexanol, 2-ethyl-1-hexanol, 1-octanol, benzyl alcohol, 2-phenylethanol, linalool, nonanal and phenylacetaldehyde. These representative volatile standards were used to imitate the real grape juice and to make the quantification accurate, because these abundant volatiles could greatly affect the SPME's adsorptive ability for MPs (Zhang *et al*., 2011). Their concentrations were set with reference to their levels in grape juice. The model solution was adjusted to pH 3.3 with 5 M of NaOH solution. Thereafter, different volumes of IBMP and IPMP stock solution (dissolved with ethanol) were added to the model solution to generate seven concentrations of MPs: 5, 25, 50, 100, 250, 500 and 750 ng/L respectively. MPs of each concentration in the model solution were extracted and analysed under the same conditions as grape juice samples. Calibration curves for IBMP ($y = 146\,460x - 32.301$) and IPMP ($y = 145\,452x - 0.4502$) were obtained via linear regression of the area ratio (124/45 for IBMP/I.S and 137/45 for IPMP/I.S) against their concentrations, with linear ranges of 10.43 - 750 and 12.41 - 750 ng/L respectively and both with regression coefficients of 0.990. The limit of detection (LOD) of these two compounds was estimated according to the method of Galvan *et al*. (2008) to be 3.16 and 3.76 ng/L respectively. To validate the analytical methodology, we added the MPs' ethanol solution into the juice of 16-week-old grapes from Huailai, up to a final concentration of 50 ng/L. SPME-GC/MS procedures were followed for five replicates. The coefficient of variation (CV) of IBMP was 6.7% and that of IPMP was 8.9%, which indicates that this method has good repeatability. The recovery of IBMP and IPMP was 108.8% and 112.2% respectively. These figures above verify that the SPME-GC/MS method used here is feasible for the determination of MPs in grape berries. Odour activity values (OAVs) were calculated by dividing the concentration by its perception threshold value from the literature.

Statistical procedures

All statistical procedures were performed using the SPSS statistical package for Windows version 16.0 (SPSS Inc., USA). A one-way analysis of variance (ANOVA) was used to gauge differences between the means of the amount of MPs, employing Duncan's multiple range tests at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Development of MPs in Cabernet Sauvignon grapes

Table 1 lists the changes in total soluble solids and pH of Cabernet Sauvignon grapes at sampling, and grapes from Gaotai, Huailai and Changli Counties on the completion of véraison at 11, 10 and 12 weeks respectively.

Only one kind of MP, namely IBMP, was detected in all the Cabernet Sauvignon samples at different developmental stages from the three producing areas considered in this study (Fig. 2).

The content of IBMP in Cabernet Sauvignon grapes from these producing areas all showed a similar trend, with a quick increase at pre-véraison and a sharp decrease in the late developmental stage. This trend was in good agreement with that described in previous reports (Ryona *et al*., 2008; Dunlevy *et al*., 2010). Similar levels of IBMP were observed in Cabernet Sauvignon grapes from the Huailai and Changli areas, and their highest contents were 129.9 ng/L and 130.6 ng/l respectively. As for the grapes from Gaotai County, the highest IBMP content reached was 175.6 ng/L, which was much higher than that in the other two areas. Meanwhile, the peak came at eight weeks after flowering, which lagged two weeks behind the IBMP peak in grapes from Huailai and Changli. Even when the grapes from Huailai County (at 14 weeks after flowering), Changli County (16 weeks after flowering) and Gaotai County (15 weeks after flowering) showed similar contents of total soluble solid (TTS) (Table 1), the samples from Gaotai County still possessed significantly the highest IBMP content, namely 32.7 ng/L, which was much higher than its perception threshold in water (2 ng/L).

No IBMP was detected in the grapes from Huailai County.

Temperature has been considered to be one of the key factors affecting the levels of MPs in grapes, and the grapevines growing in cooler climates tend to produce higher amounts of MPs than those growing in warmer climates (Lacey *et al*., 1991; Belancic & Agosin, 2007). Figure 1 shows the average temperatures of these producing areas in 2010. The active accumulated temperatures (≥ 10°C) (until harvest) in 2010 were 1 675°C, 1 784°C and 2 042°C for Gaotai, Huailai and Changli County respectively. The high IBMP levels in Gaotai grapes may be attributed to the lower active accumulated temperatures because of an early harvest, and the total soluble solids in Gaotai Cabernet Sauvignon grapes also showed much lower levels than those from the other two producing areas at harvest (Cabernet Sauvignon grapes in Gaotai County were harvested so early because low temperature came early according to historical records). The results of this study indicate that Cabernet Sauvignon grapes in Gaotai County can be harvested later by one or two weeks according to the drop in temperature so as to reduce the heavy herbaceous odour of this variety.

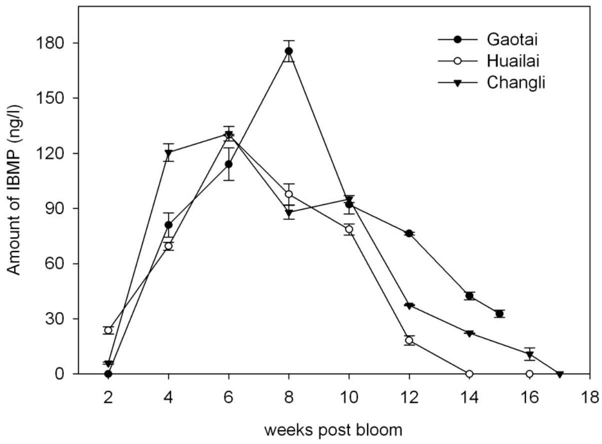

FIGURE 2
Changes in 3-isobutyl-2-methoxypyrazine in Cabernet Sauvignon grapes during berry development in Gaotai, Huailai and Changli County, China.

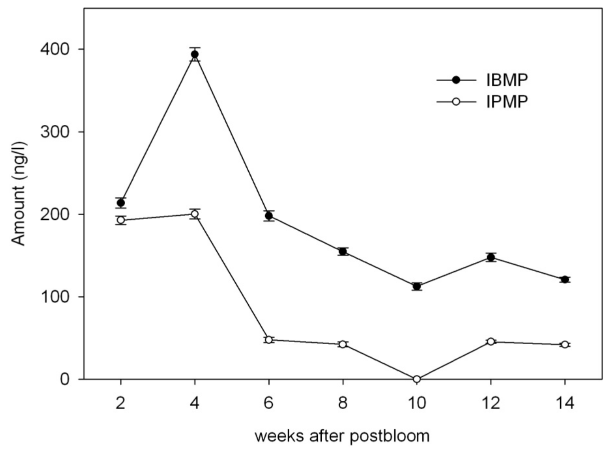

FIGURE 3
Changes in 3-isobutyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine in Zuoshanyi (*Vitis amurensis* Rupr.) grapes during berry development in Beijing.

MPs in Zuoshanyi

Two kinds of MPs, IBMP and IPMP, were detected in developing Zuoshanyi grapes and their changing trends are shown in Fig. 3.

These two MPs accumulated at the early development stage in Zuoshanyi grapes and reached a peak in four-week-old fruits, and then decreased quickly in the next two weeks until ripening. The content of IBMP in Zuoshanyi grapes was found to be much higher than IPMP throughout berry development. Although similar trends of MPs were present in the developing *Vitis vinifera* and *Vitis amurensis* Rupr. grapes considered in this study, Zuoshanyi grapes possessed much more IBMP – 393.8 ng/L in four-week-old fruit and 120.6 ng/L in 14-week fruit, in contrast to 200.2 ng/L and 41.8 ng/L respectively for IPMP. According to their perception threshold (2 ng/L in water), the odour activity values of IBMP and IPMP in ripe Zuoshanyi grapes were 100.1 and 20.9 respectively. This explains quite well why the processing product from this *Vitis amurensis* Rupr. variety generates such a strong smell of vegetal and green.

Besides the effect of variety, the low active accumulated temperature (1 811°C) is supposed to be another reason for such high levels of MPs in Zuoshanyi grapes. This variety is usually harvested early in the northeast of China because there is not sufficient temperature for use during berry development. In Beijing on the other hand, there is enough heat to be utilised for the ripening of Zuoshanyi grapes and it is suggested that the harvest date is delayed for this cultivar to reduce MP levels in the grapes and in the final wines.

CONCLUSIONS

IBMP in Cabernet Sauvignon grapes from different producing areas in Northern China showed similar trends during berry development, while its peak content in Cabernet Sauvignon grapes from cool climate areas lagged behind those from warmer areas. Zuoshanyi grapes contained much more IBMP than Cabernet Sauvignon grapes, which might explain the stronger 'green' and 'herbaceous' characters in Zuoshanyi grapes. A delay in the time of harvest for Cabernet Sauvignon in Gaotai County and Zuoshanyi in Beijing is suggested in order to reduce MP levels and the herbaceous character of these grapes.

LITERATURE CITED

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

Canuti, V., Conversano, M., Calzi, M.L., Heymann, H., Matthews, M.A. & Ebeler, S.E., 2009. Headspace solid-phase microextraction-gas chromatography-mass spectrometry for profiling free volatile compounds in Cabernet Sauvignon grapes and wines. J. Chromatogr. A. 1216, 3012-3022.

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

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

Dunlevy, J.D., Kalua, C.M., Keyzers, R.A. & Boss, P.K., 2009. The production of flavour & aroma compounds in grape berries. In: Roubelakis-Angelakis, K.A. (ed). Grapevine molecular physiology & biotechnology. Springer Science, Berlin. pp. 326 – 340.

Dunlevy, J.D., Soole, K.L., Perkins, M.V., Dennis, E.G., Keyzers, R.A., Kalua, C.M. & Boss, P.K., 2010. Two O-methyltransferases involved in the biosynthesis of methoxypyrazines: grape-derived aroma compounds important to wine flavour. Plant Mol. Biol. 74, 77-89.

Falcão, L.D., De Revel, G., Perello, M.C., Moutsiou, A., Zanus, M.C. & Bordignon-Luiz, M.T., 2007. A survey of seasonal temperatures and vineyard altitude influences on 2-methoxy-3-isobutylpyrazine, C13-norisoprenoids, and the sensory profile of Brazilian Cabernet Sauvignon wines. J. Agric. Food Chem. 55, 3605-3612.

Galvan, T.L., Kells, S. & Hutchison, W.D., 2008. Determination of 3-alkyl-2-methoxypyrazines in lady beetle-infes. J. Agric. Food Chem. 56, 1065-1071.

Hashizume, K., & Samuta, T., 1997. Grape maturity and light exposure affect berry methoxypyrazine concentration. Am. J. Enol. Vitic. 50, 194-198.

He, P. & Chao, W., 1982. Studies on the cold hardiness of eight species of *Vitis* L. growing wild in China. Acta Horticulturae Sinica 3, 17-21 (in Chinese with English abstract).

Jin, Z., He, J., Bi, H., Cui, X. & Duan, C., 2009. Phenolic compound profiles in berry skins from nine red wine grape cultivars in northwest China. Molecules 14, 4922-4935.

Koch, A., Doyle, C.L., Matthews, M.A., Williams, L.E. & Ebeler, S.E., 2010. 2-Methoxy-3-isobutylpyrazine in grape berries and its dependence on genotype. Phytochemistry 71, 2190-2198.

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

Parr, W.V., Green, J.A., White, K.G. & Sherlock, R.R., 2007. The distinctive flavour of New Zealand Sauvignon blanc: sensory characterisation by wine professionals. Food Qual. Pre. 18, 849-861.

Pickering, G., Lin, J., Riesen, R., Reynolds, A., Brindle, I. & Soleas, G., 2004. Influence of *Harmonia axyridis* on the sensory properties of white and red wine. Am. J. Enol. Vitic. 55, 153-159.

Ryona, I., Leclerc, S. & Sacks, G.L., 2010. Correlation of 3-isobutyl-2-methoxypyrazine to 3-isobuyl-2-hydroxypyrazine during maturation of bell pepper (*Capsicum annuum*) and wine grapes (*Vitis vinifera*). J. Agric. Food Chem. 58, 9723-9730.

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

Sala, C., Busto, O., Guasch, J. & Zamora, F., 2005. Contents of 3-alkyl-2-methoxypyrazines in must and wines from *Vitis vinifera* variety Cabernet Sauvignon: influence of irrigation and plantation density. J. Sci. Food Agric. 85, 1131-1136.

Sala, C., Busto, O., Guasch, J. & Zamora, F., 2004. Influence of vine training and sunlight exposure on the 3-alkyl-2-methoxypyrazines content in musts and wines from the *Vitis vinifera* variety Cabernet Sauvignon. J. Agric. Food Chem. 52, 3492-3497.

Sala, C., Mestres, M., Martí, M.P., Busto, O. & Guasch, J., 2002. Headspace solid phase microextraction analysis of 3-alkyl-2-methoxypyrazines in wines. J. Chromatogr. A. 953, 1-6.

Wu, Y., Pan, Q., Qu, W. & Duan, C., 2009. Comparison of volatile profiles of nine litchi (*Litchi chinensis* Sonn.) cultivars from Southern China. J. Agric. Food Chem. 57, 9676-9681.

Zhang, M., Pan, Q., Yan, G. & Duan, C., 2011. Using headspace solid phase micro-extraction for aromatic compounds during alcoholic fermentation of red wine. Food Chem. 125, 743-749.

Zhao, Q., Duan, C. & Wang, J., 2010. Anthocyanins profile of grape berries of *Vitis amurensis*, its hybrids and their wines. Int. J. Mol. Sci. 11, 2212-2228.