Determination of 3-alkyl-2-methoxypyrazines in greek wines and must ; the effect of temperature.

Marianthi Basalekou[1], Nikos Kalogeropoulos[2], Yorgos Kotseridis*[1]

1: Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece 2: Harokopion University of Athens, Eleftheriou Venizelou 70, Kallithea 176 76, Greece

* Corresponding author: Yorgos Kotseridis
Department of Food Science & Technology, Agricultural University of Athens,
75 Iera Odos, 11855 Athens, Greece
Tel.: +30 210 529 4702
Fax: +30 210 529 4702
e-mail address: ykotseridis@aua.gr

Abstract

The 3-alkyl-2-methoxypyrazines or MPs (IBMP, IPMP, sec-BMP and DMMP) are a group of volatile compounds, with characteristic vegetative aromas. They are mostly found in grapes of the Cabernet family, and in the hemolymph of a ladybug used to biologically control aphids. The incorporation of ladybeetles into the wine during the winemaking process causes a wine fault known as Ladybug taint (LBT). Their detection in the Greek variety of Xinomavro gives new interest to the analysis of Greek wines, for which no data are available, while the rapid spread of Harmonia axyridis in Europe urges the development of a method for simultaneous detection of all four methoxypyrazines in order to control their levels during the winemaking process. In the present study methoxypyrazines were detected and quantified in Greek grape juice and wine through the method of solid phase extraction (SPE), using columns with different packings, one of which allowed the simultaneous detection of all four methoxypyrazines. Of the wines analyzed, only one sample of Xinomavro showed MP limits above the methods' detection threshold. Furthermore, the application of high levels of temperature (50 ˚C) appeared to reduce levels of methoxypyrazines up to 87%, thus highlighting thermovinification as a potential method for their control during winemaking.

1. Introduction

3-alkyl-2-methoxypyrazines (MPs) are nitrogen-containing compounds with green aromas, mainly found in grapes and wines from the Vitis Vinifera varieties Cabernet sauvignon (Bayonove et al, 1975), Cabernet franc, Sauvignon blanc and Merlot (Harris et al. 1987, Allen et al. 1994, Hashizume and Umeda 1996, Kotseridis et al. 1998), and also in the hemolymph of certain species of ladybugs (mainly Harmonia axyridis) (Fig.1)[1]. They are characterized by their high aromatic intensity and extremely low sensory thresholds (ng/L)[2]. The four main methoxypyrazines found in wines are: 3-isopropyl-2-methoxypyrazine (IPMP), 3-isoboutyl-2-methoxypyrazine (IBMP), 3-sec-boutyl-2-methoxypyrazine (secBMP) and 2,5-dimethyl-3-methoxypyrazine (DMMP). The most important methoxypyrazine is considered to be IBMP, a methoxypyrazine that exhibits the classic green bell pepper aroma of Sauvignon blanc wines and is also found in higher concentrations than the others[3].

The final MP concentration in grapes and subsequently wine, depends on the climate, the viticultural techniques and the winemaking protocol. Higher concentrations of MPs are found in

cold climates, high altitude vineyards as well as in organically grown ones, due partly to the use of ladybugs as biological controlling agents which explains their increased populations there[4,5,]. The main tendency is to keep their presence at a minimum, and that is because except for winemaking regions like South Africa or New Zealand, they can alter a wines' organoleptic profile drastically., In the case that MPs originate from the hemolymph of Harmonia axyridis, the dominant MP is IPMP and the aromas it imparts into the wine are more moldy, peanutbutter-like ones and not so much grassy or even desirable aromas[6]. The incorporation of ladybugs into a fermenting grape juice alters not only its aroma, but its taste also, as the ladybug's hemolyph contains lots of alcaloids, which are excessively bitter[7].

Methoxypyrazine levels can be controlled in the vineyard, via extending maturation or with defoliation, or in the winery, where the methods proposed for their elimination are filtration or blending, with not very good results until now.

Methoxypyrazines' low sensory thresholds can also be a big challenge regarding their analysis. Due to their volatility, gas chromatography has been commonly employed. Regarding sample preparation, Head Space-Solid Phase MicroExtraction (HS-SPME)[8] has given some good results in wines and is preferred due to its selectivity and speed. Recently, Solid Phase Extraction with the use of ethylvinylbenzene-divinylbenzene columns have made MPs identification possible to the point of picograms per litre (GC-MS-MS)[9].

Our goal was firstly to develop a fast clean up procedure based on SPE and coupled with GC-MS that could help detect or identify MPs potentially also in greek wines given the detection of IBMP in a wine from the greek grape variety Xinomavro some years ago, and secondly to evaluate the effectiveness of applying high temperatures to the must to control the concentration of MPs.

2. Materials and methods

For the identification and quantification of MPs in must and wine a solid phase extraction (SPE) methodology was established, based on methods proposed by Pickering et al., (2005)[10] and Ferreira et al., (2009)[9] . The compounds analyzed were IBMP, IPMP, secBMP and DMMP, using their respective deuterated analogues ([$^2H_3$]-IBMP, [$^2H_3$]-IPMP, [$^2H_3$]-secBMP, [$^2H_3$]-DMMP) or d-MPs as internal standards. All analysis were carried out at a gas chromatograph coupled with mass spectrometry.

2.1.Standards and solutions

Reference standards used for identification were purchased in pure form (99%) from Sigma Aldrich and included 2-methoxy-3-isobutylpyrazine (IBMP), 2-methoxy-3-isopropylpyrazine (IPMP), 2-methoxy-3-(1-methylpropyl)pyrazine (secBMP). DMMP and all MPs' deuterated analogues were obtained by the laboratories of Cool Climate Oenologie and Viticulture Institute at Ontario, Canada where they were prepared following the methods proposed by Seifert et al., (1970)[11] and Kotseridis et al., (1998)[12]. The four standards (IBMP, IPMP, secBMP, DMMP) were used for quantification of the amount of MPs. An individual standart solution of 1 g/L of each MP was prepared in certified A.C.S.-grade ethanol (Sigma). One combined standard solution containing all the MPs and one with their deuterated analogues was also prepared and subsequently diluted with ethyl acetate to 1μg/L, and finally stored in dark at -4ºC. Standard solutions in further studies were prepared fresh by diluting different amounts of the standard solutions with ethyl acetate to the required concentrations.

2.2 Wine and juice samples

All juice and wine samples were obtained from the greek market. They were from indigenous grape

varieties, most of which were grown organically or in high altitude vineyards. The greek grape varieties chosen are: Roditis, Debina (whites), Mavroudi, Vlahiko, and Xinomavro (reds). Two wines from Xinomavro were analysed, samples Xinomavro K and Xinomavro F, both from vineyards cultivated in Northern Greece, one of which (Xinomavro K) was cultivated organically.

2.3 Instrumental and data analysis

The samples were analysed with an Agilent 6890N GC / 5973N MSD equipped with a 5 % Phenyl Methyl Siloxane column (30m, 0.24 mm id, 0.25µm film thickness). Helium was used as the carrier gas with a nominal initial flow of 1 ml/min. The oven was held at 40ºC for 3 minutes, then increased to 115ºC for 2 minutes, and finally increased to 240ºC for 1 minute. The injector and detector temperatures were 250ºC and 300ºC respectively. Identification was achieved using Selected Ion Monitoring (SIM), with 3µl splitless injection of the sample for 30 sec. For IBMP selected mass channels were *m/z* 124 and 151 and *m/z* 127 and 155 for [$^2H_3$]-IBMP. Ions 124 and 127 were used for quantification (Fig. 2a), while ions 151 and 155 were used as qualifier ions. For IPMP, selected mass channels were *m/z* 137 and 152 and *m/z* 140 and 155 for [$^2H_3$]-IPMP. Ions 137 and 140 were used for quantification, while ions 155 and 152 were used as qualifier ions. For sBMP, selected mass channels were *m/z* 138, 151 and 124, and *m/z* 141, 127 and 154 for [$^2H_3$]-sBMP. Ions 138 and 141 were used for quantification, while ions 151, 124, 127 and 154 were used as qualifier ions. For DMMP, selected mass channels were *m/z* 138, 123 and 137 and *m/z* 145 and 144 for [$^2H_3$]-DMMP. Ions 138 and 145 were used for quantification, while ions 144, 123 and 137 were used as qualifier ions. All samples were analysed in duplicate or triplicate. After each set of samples a fixed concentration sample containing all standards along with their respective deuterated ones was analysed so as to check for contamination of the fibre.

Compounds were identified by matching of the retention time on the GC capillary column with the retention time of pure compounds run as standards, and by matching mass spectrums of unknown compounds with MS library search system and spectrums of pure compounds (Fig. 2.3).

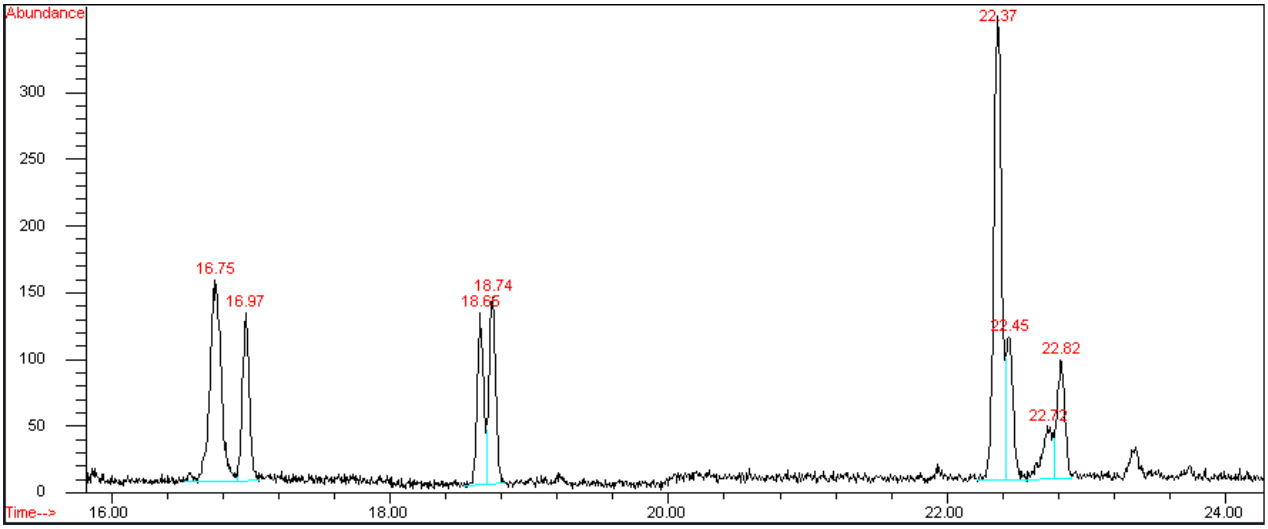

Figure 2.3 Chromatogram of a standard solution containing all MPs in 10µg/L concentration. Each MP's retention time is marked on top of its respective peak.

2.4 SPE method development

The following SPE parameters have been optimized during method development : activation solutions volumes, sample pH and volume content, clean up, elution and concentration procedures.

For C-18 columns, alterations were made on the method proposed by Pickering et al. (2005) which included the following steps:100mg C-18 columns in 3ml cartridges are conditioned by 1ml ethyl acetate, 1ml of 95% ethanol and 1ml 10% ethanol, following 50 ml of filtered wine, 10 minute wait to let the sorbent dry and finally elution with 1ml dichloromethane.
For ethylvinylbenzene- divinylbenzene packing, the columns were Lichrolut's EN columns, and the method proposed by Ferreira et al., (2009) included the following steps: 200 mg Lichrolut EN columns in 3 ml cartridges are conditioned by 10 ml dichloromethane, 10 ml methanol and 10 ml 15% ethanol, after which 100 ml of filtered wine is passed through the column, then 50 ml of an aqueous solution of 50% methanol and 1% $NaHCO_3$ to clean the column, and finally after 10 minutes to allow drying of the sorbent, elution is made by 1,5 ml of dichloromethane.

2.5 Calibration curves and detection limits.

A MP-free wine of 12% (v/v) ethanol was used for the calibration curves. The wine was spiked with MPs to give MPs concentrations in the range of 15-60 ng/L. Each sample contained also fixed concentrations of d-MPs (700ng/L d-DMMP, 100ng/L d-IPMP, 300 g/L d-secBMP and 200 ng/L d-IBMP). The detection limits of the method was 15 ng/L for both types of columns regarding all the MPs except DMMP which could not be quantified, whereas the detection limit for the C-18 modified method regarding IPMP, was 5 ng/L (Fig. 2.5). The estimation of the detection limits was based on the signal to noise ratio.

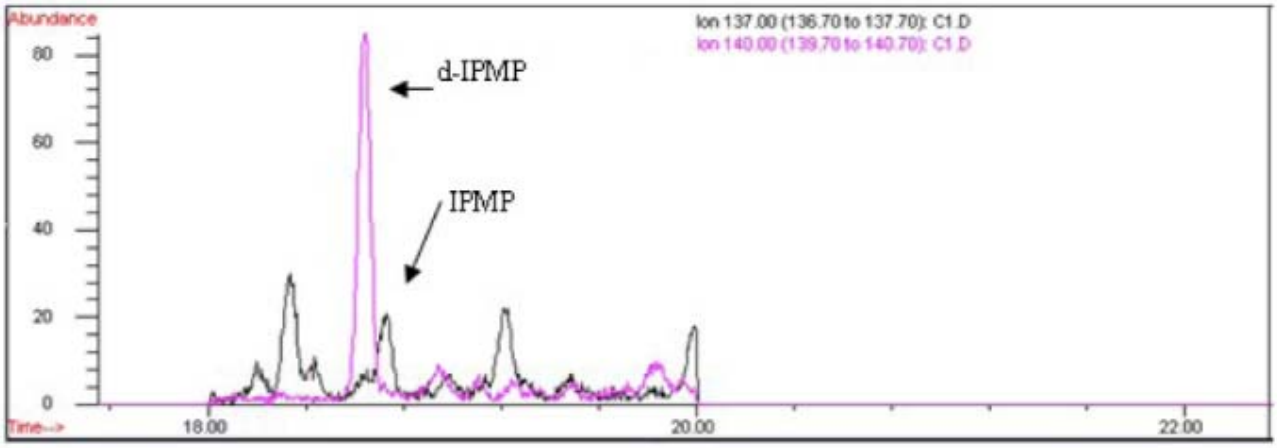

Figure 2.5 Chromatogram of IPMP at 5 ng/L concentration after C-18 modified method including concentration of the sample under nitrogen stream.

2.6 **Temperature effect**

To evaluate the effect of temperature on the concentration of MPs, 1 L of red grape must was spiked with 40 ng/L of each methoxypyrazine after the must had been filtered, and it was divided to 9 erlenmeyer flasks (100 ml of spiked must in each flask). Three of these flasks were placed in an oven set at 60ºC where they remained for 6 hours without their lid on. Another set of three flasks with their lids on were placed in the same oven, and also stayed for 6 hours, while the last three remained in room temperature for 6 hours without a lid. After the 6 hour wait, the deuterated MPs were added to each flask, and they were all analysed.

3. Results and discussion

3.1 SPE modifications.

From the modifications made on Pickering and Ferreira methods, the most notable results were obtained by changing the sample's pH and alcohol content and by concentrating the elute

under nitrogen stream. The shift in pH promotes the retention of the MPs, which are slightly basic compounds from the silanoid (Si-O) groups of the sorbent in C-18 columns, while the dilution of the samples' regarding its' alcohol concentration minimises the antagonism between ethanol and C-18 to retain the MPs. Both modifications gave chromatograms with less noise and clearer peaks (Fig. 3.1a). For the elution of the MPs both dichloromethane and ethyl acetate were tried, but only dichloromethane gave chromatograms with clearer MP peaks after concentration under nitrogen stream, probably because MPs were drifted away along with ethyl acetate as it is less volatile (Fig. 3.1b).

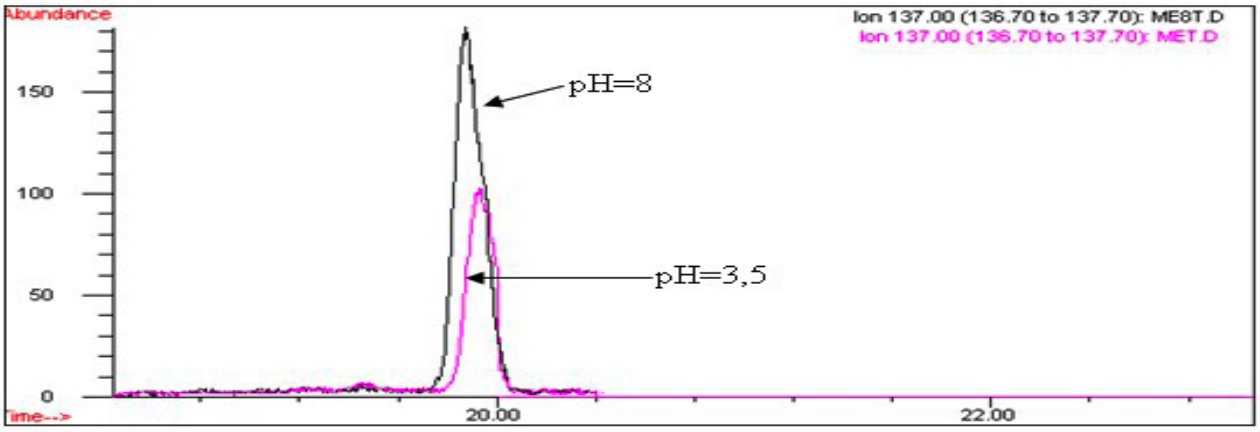

Figure 3.1a Indicative chromatogram for IPMP with sample pH adjustment from pH 3,5 to pH 8

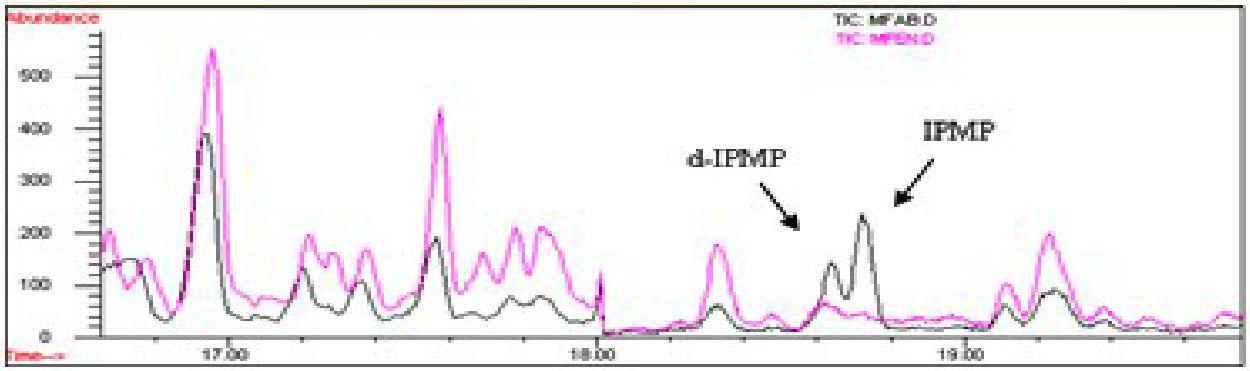

Figure 3.1b Indicative chromatogram for IPMP showing samples eluted with DM and EtAc. IPMP is identifiable only when it is eluted with DM. Both samples were concentrated under nitrogen stream to a final volume of 100μl.

For the Lichrolut EN columns the most decisive modification step was the clean up of the sorbent bed. Clearer chromatograms were obtained by doubling the clean-up solutions' volume especially in red wines that had higher concentration of co-eluting compounds to remove (Fig. 3.1c).

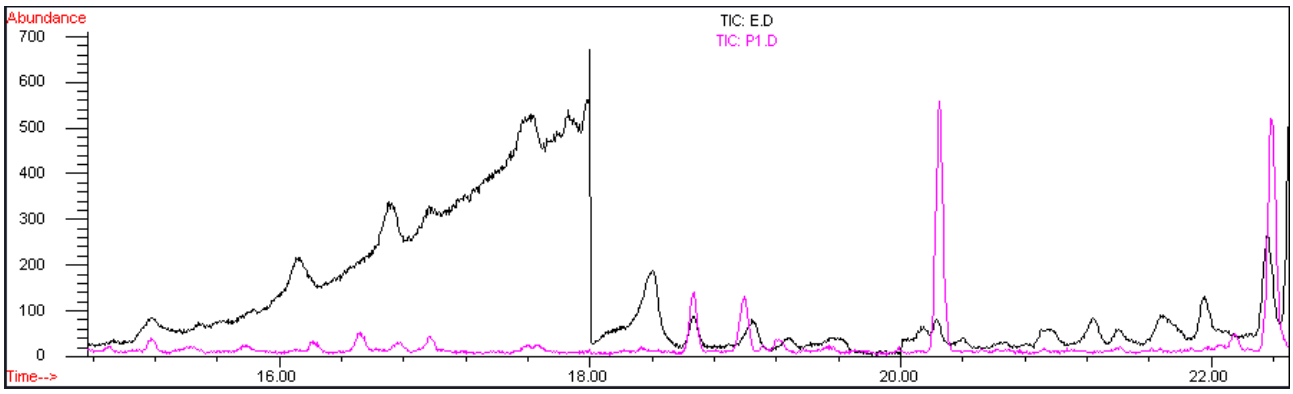

Figure 3.1c Overlapping chromatograms of samples were the clean-up solvent's volume were 25 and 50 ml. The clear peaks with less noise belong to the sample which was treated with the 50 ml.

3.2 Identification and quantification of MPs in wine samples

Of the wines analysed, methoxypyrazines were detected in three wine samples, one from the greek grape variety Mavroudi, and two from the Xinomavro variety. Only in one of the Xinomavro samples MPs were quantified, namely IPMP, and its' concentration was found to be 40 ng/L (Table 3.2). The SPE method used for the analysis was the C-18 modified one.

Table 3.2 Methoxypyrazine concentrations in wine samples.

Wine Sample	Methoxypyrazine concentration (ng/L)			
	DMMP	IPMP	secBMP	IBMP
Roditis	nd	nd	nd	nd
Mavroudi	nd	nd	nd	<15
Vlahiko	nd	nd	nd	nd
Debina	nd	nd	nd	nd
Xinomavro K	nd	41	nd	nd
Xinomavro F	nd	<15	nd	nd

3.3 Effect of temperature

The levels of all three methoxypyrazines (DMMP was not added due to difficulties in its quantification) that have been added to the juice that stayed for 6 hours in the oven showed a decrease ranging from 74% (IBMP) to 87,5% (IPMP) (Fig. 3.3, Table 3.3). This decrease was observed only in the case of the heated flasks without lids, while the ones that were heated with their lids on showed no change at their MP concentration. This leads to the conclusion that the level of MPs in the samples decreased due to their evaporation.

Table 3.3 % Decrease in MPs levels after heating at 60ºC for 6 hours

Methoxypyrazine	% decrease after heating
IPMP	87.5
secBMP	80
IBMP	74

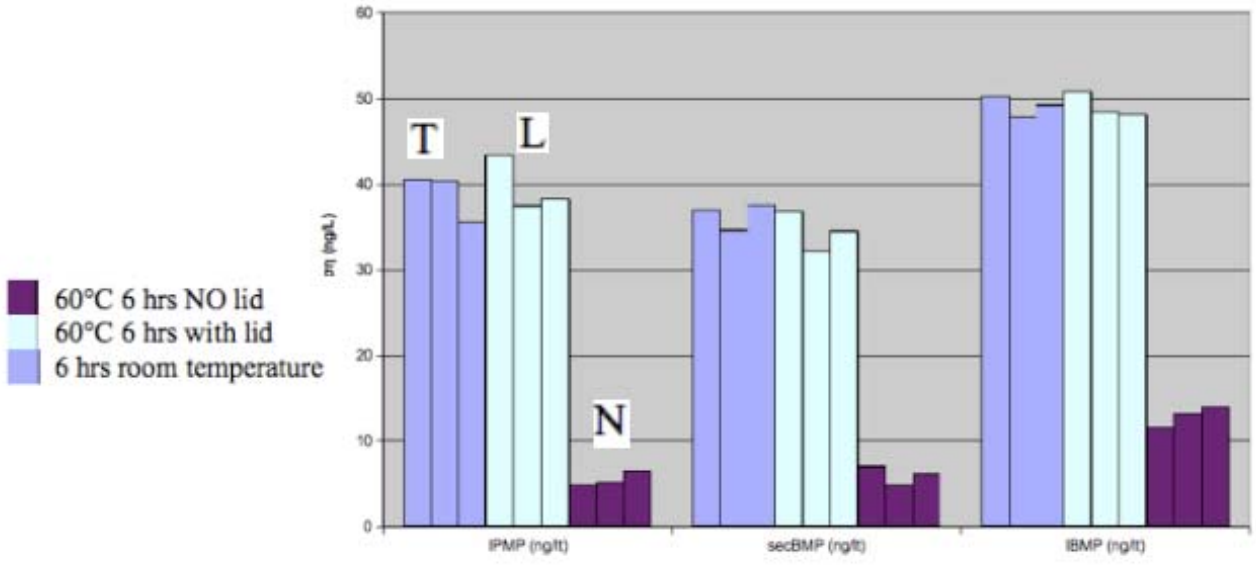

Figure 3.3 Effect of temperature on Methoxypyrazine spiked samples heated with no lid (N samples), with lid (L samples) at 60ºC for 6 hours, or stayed with no lid at room temperature for 6 hours (T samples)

4. Conclusions

Two SPE methods were modified for the quanification and identification of methoxypyrazines in grape juice and wine. In the case of the method using C-18 columns, pH adjustion to pH 8 and concentration of the elute from 1ml to 100μl under nitrogen stream allowed the identification of all MPs and quantification of IPMP and IBMP. The detection limit was 5ng/l for IPMP, and 15 ng/L for all the other MPs. For the Lichrolut EN columns, doubling the clean up solvent volume allowed the identification of all four MPs and the quantification of IPMP, IBMP and secBMP to a detection limit of 15 ng/L. Using the Lichrolut EN method, IBMP and IPMP were detected in three samples of greek wines, and IPMP was also quantified in a wine from Xinomavro. Although this observation requires further experiments, the detection of IPMP in this relatively high concentration can be linked with the presence of ladybeetles in the organically grown vineyard from which the grapes for the Xinomavro wine originated.

The decrease in the levels of MPs by 74-87,5%, highlights the use of thermovinification as a potential method to lower methoxypyrazine concentrations in wines.

Acknowledgements

Thanks are due to Harokopion University where the GC-MS analysis of the grape juice and wine samples was carried out. Special thanks to the Cool Climate Oenologie and Viticulture Institute at Ontario, Canada for providing the internal MP standards.

References

1. Lingshuang Cai, Jacek A. Koziel and Matthew E. O'Neal, Determination of characteristic odorants from Harmonia axyridis beetles using in vivo solid-phase microextraction and multidimensional gas chromatography–mass spectrometry–olfactometry, Journal of

Chromatography A, Volume 1147, Issue 1, 13 April 2007, Pages 66-78

2. G.J. Pickering , A. Karthik , D. Inglis , M. Sears , K. Ker Determination of Orthoand Retronasal Detection Thresholds for 2-Isopropyl-3-Methoxypyrazine in Wine. Journal of Food Science Volume 72 Issue 7, Pages S468 – S472

3. Sala C, Bustoa O., Guasch J, Zamora F. Factors affecting the presence of 3-alkyl- 2-methoxypyrazines in grapes and wines. A review.

4. C. Ross, K. Weller, Sensory evaluation of suspected Harmonia axyridis-tainted red wine using untrained panelists. Journal of Wine Research, Vol. 18, No. 3, pp. 187-193, 2007.

5. Abassi S. Al., Birkett M. A., Petterson j., Pickett J. A., Woodcock C. M., Ladybird beetle odour identified and found to be responsible for attraction between adults. CMLS, Cell. Mol. Life Sci. 54 (1998) 876-879

6. E. Lucas, I Gagne, D. Coderre, Impact of the arrival of Harmonia axyridis on adults of Coccinella septempunctata and Coleomegilla maculata (Coleoptera: Coccinellidae). Eur. J. Entomol. 99: 457-463, 2002.

7. G. Pickering, J. Lin, R. Riesen, A. Reynolds, I. Brindle, G. Soleas Influence of Harmonia axyridis on the Sensory Properties of White and Red Wine Am. J. Enol. Vitic. 55:2:153-159 (2004).

8. Sala C, Bustoa O., Guasch J. Determination of 3-alkyl-2-methoxypyrazines in grapes, musts and wines: a review.

9. Laura Culleré, Ana Escudero, Eva Campo, Juan Cacho and Vicente Ferreira Multidimensional gas chromatography–mass spectrometry determination of 3-alkyl-2- methoxypyrazines in wine and must. A comparison of solid-phase extraction and headspace solid-phase extraction methods Journal of Chromatography A, Volume 1216, Issue 18, 1 May 2009, Pages 4040-4045

10. Gary J. Pickering, Yong Lin, Andrew Reynolds, George Soleas, Roland Riesen, Ian Brindle,The Influence of Harmonia axyridis on Wine Composition and Aging. Journal of Food Science Volume 70, Issue 2 , PagesS128 - S135 2005

11. Seifert R. M., Buttery R. G., Guadagni D. G., Black D. R., Harris J. G., Synthesis of Some 2-Methoxy-3-Alkylpyrazines with Strong Bell Pepper-Like Odors. J. Agr. Food Chem., Vol. 18, No.2, 1970

12. Kotseridis, Y.; Baumes, R.; Skouroumounis, G. K. Synthesis of labelled [2H4] β- damascenone, [2H2] 2-methoxy-3-isobutylpyrazine, [2H3] α-ionone, and [2H3] β- ionone, for quantification in grapes, juices and wines. J. Chromatogr. A. 1998,824, 71-78.