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**INVESTIGATIONS OF 2-ALKYL-3-METHOXPYRAZINES IN
THREE LADYBUG SPECIES AND WINE**

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A thesis submitted to the Department of Chemistry in partial fulfillment of the
requirements for the award of the Master of Science degree

Brock University
St. Catharines, Ontario

May, 2005

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Investigations of 2-alkyl-3-methoxypyrazines in three ladybugs species and wine

Abstract

Investigations of 2-alkyl-3-methoxypyrazines (2-isopropyl-3-methoxypyrazine, 2-secbutyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine) in ladybug species (*Coleoptera: Coccinellidae*) and wine samples have been conducted. Headspace sampling coupled with gas chromatography-mass spectrometry was used to determine amounts of 2-alkyl-3-methoxypyrazines in the ladybug species. *Hippodamia convergens* had the highest amount of alkylmethoxypyrazines, followed by *Harmonia axyridis* and the least in *Coccinella septempunctata*.

Using a solvent extraction method, the precoccinelline alkaloid was found present in *Hippodamia convergens* and *Coccinella septempunctata* but not *Harmonia axyridis*.

Steam distillation followed by a solid phase extraction method as a sample preparation technique, enhanced detection while the isotope dilution method afforded accurate quantitation of the alkylmethoxypyrazines in the wine samples. Both ladybug-tainted and commercial wine samples were found to contain the 2-alkyl-3-methoxypyrazines. Wine samples prepared in 2001 generally contained higher levels than the corresponding 2003 samples. Levels of the 2-alkyl-3-methoxypyrazines found in the commercial wines ranged from a minimum value of 6 ng/L to 260 ± 10 ng/L. Analyses revealed that for both ladybug species and wine samples, the 2-isopropyl-3-methoxypyrazine had the highest concentration, followed by 2-isobutyl-3-methoxypyrazine and the least being the 2-secbutyl-3-methoxypyrazine. Possible contamination of the wine samples by ladybugs is thoroughly discussed.

Furthermore, attempts to remove or reduce the levels of the alkylmethoxypyrazines with molecularly imprinted polymers from wine samples are presented in detail.

Acknowledgements

First and foremost, I wish to express my sincere gratitude to the good Lord for sustaining my life up to this level of my academic endeavours.

I am indebted to numerous persons who have offered help to me in this work.

I am particularly grateful to Professor Ian Brindle, for his ever abounding patience, altruistic dedication to this work and for sharing his scientific perspective which unfolded a new world of ideas to me and for all the numerous things he has given me all through this thesis.

Thanks to Roger McLaughlin for his guidance and support throughout this work. I would also want to show appreciation for the expert recommendations I received from my committee members.

Thanks are due also to Tim Jones and Donna Vukmanic for their immense help on the gas chromatography mass spectrometry training. To Dr. Gary Pickering and the entire Cool Climate Oenology and Viticulture staff, I say thanks for the excellent sensory evaluation work done and their willingness to always provide me with wine samples always. I deeply appreciate the efforts of Dr. Lu Chunyang, in the preparation of the molecularly imprinted polymers and selfless commitment to this work. My sincere appreciation also goes to Xiaonan Chen for synthesizing the deuterated 2-isopropyl-3-methoxypyrazine, which was used for the isotope dilution method.

Lastly, hearty thanks to Priscilla for her greatly needed support, care and prayers that tend to make life easier and filled with optimism. To the kids, I say bravo for the beauty you bring to our family.

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CHAPTER 1

Introduction

Pyrazine is an aromatic heterocyclic compound. Its derivatives like the methyl, ethyl, methoxy, isopropyl, isobutyl and secbutyl form the major flavour components in many foods. Maga and Sizer reviewed current knowledge of pyrazines in foods and gave a list of food materials with their corresponding type of pyrazine compound.¹

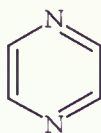
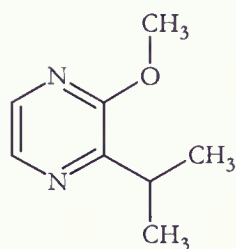
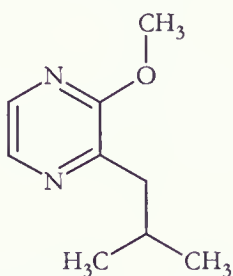


Figure 1.1 Structure of pyrazine

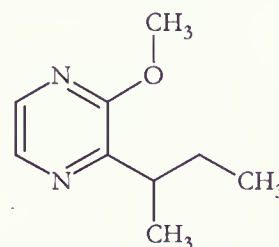
Among the many pyrazine compounds identified, the most abundant are the 2-alkyl-3-methoxypyrazines.² These compounds, 2-isopropyl-3-methoxy pyrazine (IPMP), 2-*sec*-butyl-3-methoxypyrazine (SBMP) and 2-isobutyl-3-methoxy pyrazine (IBMP) have generated a great deal of interest amongst researchers. The interest in the compounds is due to the powerful aroma and taste characteristics of these substituted pyrazines.



2-isopropyl-3-methoxypyrazine



2-isopropyl-3-methoxypyrazine



2-isopropyl-3-methoxypyrazine

Figure 1.2 Structures of 2-alkyl-3-methoxypyrazines

In water, the threshold detection of IBMP is reported as 2 ng/L.^{3,4,5} Buttery *et al.* described the green bell pepper odour characteristic of IBMP.³ Up to 20 ppm of

IBMP has also been reported in bell peppers and other foodstuffs (carrots, asparagus, beans, lettuce, chillis, etc.).⁶ In wines, particularly those from New Zealand, 2-alkyl-3-methoxypyrazines are also associated with the vegetal and herbaceous flavour of Cabernet Sauvignon, Merlot, and Sauvignon Blanc varieties.⁷ Other reports indicate the presence of these compounds in various grape varieties. There have been speculations of the 2-alkyl-3-methoxypyrazines being associated with secretions of ladybugs (Coleoptera: Coccinellidae). If these speculations can be justified, the impact of the large aggregations of these ladybug species, especially in vineyards, may have significant impact on wine quality.

Alkylmethoxypyrazines in Grapes and Wine

The alkylmethoxypyrazines, in particular the 2-alkyl-3-methoxypyrazines, are strikingly potent odorants. These volatile organic compounds are found in the berries of several grape varieties particularly those of Sauvignon Blanc, and Cabernet Sauvignon vines.⁸ Consequently these compounds are incorporated into the wines produced using these grape varieties at a concentration that can present distinctive aroma. Thus relatively high levels of the methoxypyrazines are characteristic of these grape varieties. The IBMP was first detected by Bayonove, *et al.* in Cabernet Sauvignon grape and subsequently considered to be essentially responsible for the vegetative green bell pepper aromas in some wines derived from a number of *Vitis vinifera* grape varieties (Cabernet Sauvignon, Cabernet franc, Merlot and Sauvignon Blanc).⁹ Due to their potent odour, too high a concentration of

alkylmethoxypyrazines are regarded as obnoxious, and hence damaging to wine quality. Nonetheless, at lower concentration they play a key role in distinguishing wines of these varieties from others.¹⁰ Allen and Lacey in their study of methoxypyrazine grape flavour in various regions, discovered that the concentration of IBMP in grapes tends to decrease during maturation or ripening.^{10,11} This phenomenon was attributed to the influence of climate, the vines' vegetative growth, the vineyard management techniques, and the exposure of the grapes to sunlight. The highest concentration of alkylmethoxypyrazines occurs in unripe grapes and may reach to about 100 ng/L in Sauvignon Blanc and Cabernet Sauvignon juice.¹² Under comparable climatic conditions in Bordeaux vineyards, Roujou de Boubée *et al.* reported on the importance of humidity in maintaining higher levels of alkylmethoxypyrazines during the ripening period.⁹

Alkylmethoxypyrazines are also present in the grape stem, seeds, and skin. Unlike the stem and seeds, the skins tend to show a relatively higher concentration of the alkylmethoxypyrazines even after maturation.¹³ Thus, the nature of the grapes themselves and the conditions under which they are grown could have a significant impact on the amount of alkylmethoxypyrazine in wine.

The Ladybugs and Alkylmethoxypyrazines

The name ladybird originated with the naming of the European beetle *Coccinella septempunctata*. As knowledge on insects increased, the name was extended to the family Coccinellidae. Some of these species are considered to be native to North America, while others are considered invasive species.^{14, 15}

On the average, female ladybugs are larger than males with the adult of some species being brightly coloured. The adult ladybugs are known to reflex-bleed from their joints. The blood (hemolymph) has a characteristic repulsive odour. Analyses of the hemolymph gave the alkaloid toxins adaline, coccinelline, hippodamine, among others.¹⁴ Due to the characteristic potent odour of the hemolymph and its toxicity, it is believed to serve as a defensive mechanism against predators.^{16, 17}

The Coccinellidae can be found throughout North America and in many other parts of the world. Some of the species include *Harmonia axyridis*, *Coccinella septempunctata*, *Hippodamia convergens*, *Coleomegilla maculata*, among others. These ladybugs are important predators contributing to the natural control of pest aphid (Aphididae) populations. For example, the multicoloured Asian ladybug (*Harmonia axyridis*) was deliberately introduced from its native Western Asia into North America, first in 1918, but more successfully in the 1980s, to control aphids on plants (pecan trees, pine trees, ornamental shrubs, cotton, wheat, tobacco, roses).^{14, 18} Due to their predatory characteristics, they are used for biological pest control. However, they are also attacked by a range of natural enemies, especially birds.¹⁸ The wasp *Dinocampus*

coccinellae (Hymenoptera) is known to be the major parasite and can cause considerable decrease in populations of the *Coccinella septempunctata* (7-spot ladybug).¹⁸ The alkaloids secreted from these ladybugs, especially the brightly coloured ones, are believed to be responsible for their aposematic colouration, that is, the colours warn predators that the ladybugs are repugnant or toxic.^{14, 17}

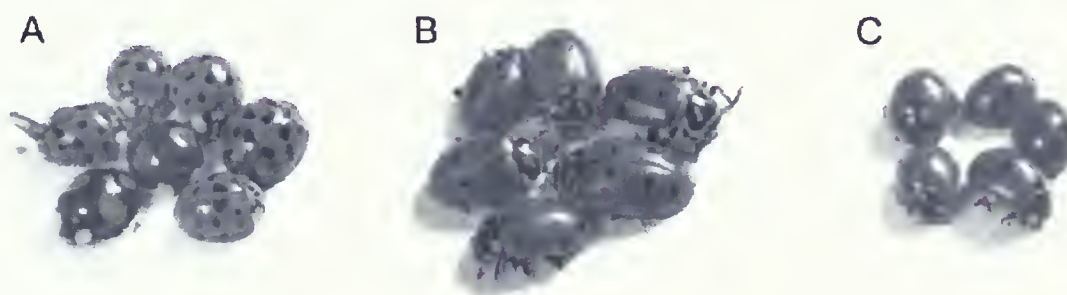


Figure 1.3 A. *Harmonia axyridis*, B. *Hippodamia convergens*, C. *Coccinella septempunctata*²⁰

More than fifty alkaloids have been isolated from the Coccinellidae.²⁰ The ladybug species investigated include *Harmonia axyridis*, *Coccinella septempunctata*, *Hippodamia convergens*, *Adalia bipunctata* among others. The alkaloid harmonine has been isolated from the *Harmonia axyridis* and *Hippodamia convergens* whereas the *Coccinella septempunctata* produces the precoccinelline and its oxide, coccinelline.¹⁷ Alam, *et al.*, in addition to harmonine, also reported the presence of 3-hydroxypiperidin-2-one in *Harmonia axyridis* and *Aiolocaria hexaspilota*.²¹ The smaller 2-spot ladybug (*Adalia bipunctata*) is known to secrete a different alkaloid known as adaline, which has a much less repulsive odour to predators than the coccinellines.²¹ As a result, the 2-spot ladybugs give off more reflex fluid in order to provide effective defense.²¹ Another important alkaloid is hippodamine, which is found in the secretions of *Hippodamia*

convergens. This alkaloid differs from that of the precoccinelline only in terms of their stereochemistry. Generally, it is suggested that most of the aliphatic nitrogen-containing alkaloids are derived from amino acids.²⁰

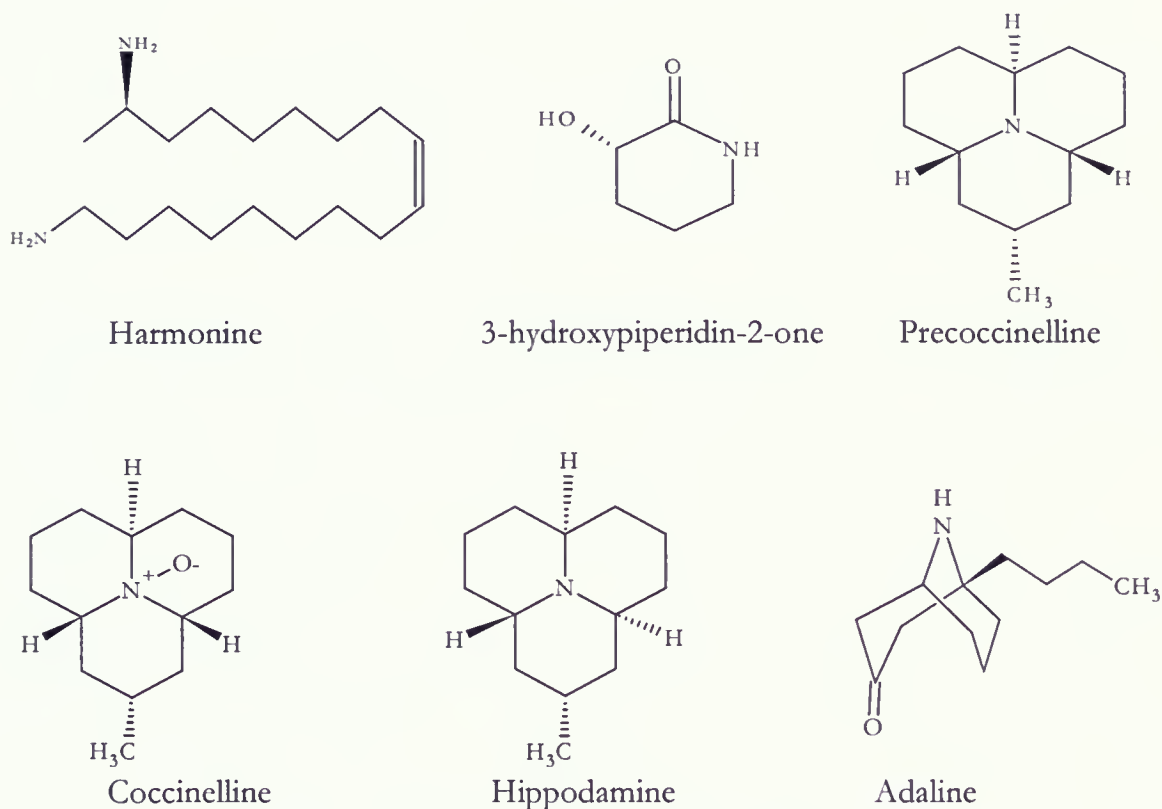


Figure 1.4 Alkaloids detected in ladybugs species

In addition to the undesirable bitter alkaloid taste that can be imparted into wine, recent reports by Ray and Pence suggest that secretions from *Harmonia axyridis* species will possibly be added to the list of inhalant allergens.¹⁶ In an abstract of the report, they indicated that there have been several cases in the literature describing patients suffering from allergic respiratory symptoms including rhinitis and asthma that are related to exposure to ladybugs. Notwithstanding, there had not been a serious health case reported as a result of one's exposure to these ladybugs.

Some species of ladybugs are also believed to secrete semiochemicals that comprise alkylmethoxypyrazines, which are commonly used as olfactory alerting signals. Probable suggestions are that IPMP, IBMP and SBMP are compounds that may be secreted by the 7-spot ladybug¹⁸ and *Harmonia axyridis*. In the late summer of 2001, Eastern North America experienced an extraordinary aggregation of large numbers of the *Harmonia axyridis* species. This huge aggregation was a result of deliberate use of *Harmonia axyridis* to control a soy aphid population explosion that had occurred.²² This led to houses and wineries being infested by significant numbers of the ladybugs during late fall.²² The possible effect of this invasion on wineries is the incorporation of their defensive olfactory secretion being imparted to must and wines.

The typical vegetative or herbaceous odour characteristic of the alkylmethoxypyrazines, which was associated with Sauvignon Blanc, Cabernet Sauvignon and Merlot wines, consequently masked to varying degrees the varietal “bouquet” of the particular grape from which the wine was made. Although, it was not certain that the noticed off-flavours of wines were due primarily to the ladybugs, recent studies by Pickering, *et al.* on the sensory properties of Riesling wines seem to confirm this as a probable cause.²³ In the light of this aggregation pattern on vineyards, and the probable contamination with alkylmethoxypyrazines, the quality and commercial value of wines may significantly be affected.

Analysis of Alkylmethoxypyrazines in Wines

Recently, several analytical methods have been developed to investigate alkylmethoxypyrazines in grapes, wines and other foodstuffs. Heymann, *et al.* used high performance liquid chromatography (HPLC) to quantify IBMP in a Chenin Blanc wine. In this analysis, steam distillation followed by pre-concentration on a C₁₈ – cartridge was used for sample preparation.²⁴ The method had poor yield ($53 \pm 7\%$) and a high detection level (1.2 µg/L). A similar analysis of IBMP in Bordeaux wine using gas chromatographic technique, gave a rather high concentration (500 ± 70 ng/L).²⁵ Taking into account the difficulties associated with accurate determination of the ultratrace amounts of the alkylmethoxypyrazines in wines, hyphenated analytical systems became a better alternative. Some of the hyphenated analytical systems used include gas chromatography mass spectrometry (GC/MS),^{7, 26, 27, 28} headspace-solid phase microextraction (HS/SPME) coupled with the GC/MS and headspace – gas chromatography-mass spectrometry (HS-GC/MS).^{20, 29, 30}

Due to the volatility of alkylmethoxypyrazines coupled with the fact that they are often in very low concentrations, the GC/MS served as the more suitable analytical technique for qualitative and quantitative analyses. With the appropriate sampling and sample preparation, the GC/MS has been used to achieve very low detection threshold (ng/L range). For example, Murray and Whitfield in 1975 analyzed the headspace volatiles above raw vegetables by GC/MS for the identification of the 2-alkyl-3-methoxypyrazines (IPMP, IBMP and SBMP) in the low ng/L levels.⁸

Various approaches have been used for sample preparation in the determination of the alkylmethoxypyrazines by GC. These approaches include solvent extraction, headspace analysis, solid phase extraction (SPE), HS/SPME, and HS. With the correct precautions these methods provide effective sample preparation. Of these methods, headspace sampling has generated significant interest among analytical chemists. This interest is due to its rapidity, convenience and amenability towards automation. Since this method does not require laborious sample preparation, the risk of analyte loss through dilution, removal of potential interferences by masking, evaporation, filtration, analytes getting glued to glassware and/or altering its original form can be reduced.

Headspace sampling is an analytical method developed for determination of volatiles and semi-volatiles in both solid and liquid matrices. Allen, *et al.* reported an example where the headspace sampler has been used to investigate 2-alkyl-3-methoxypyrazines in raw vegetables.⁸ The basis of headspace analysis (HSA) dates back to 1939 when Harger, *et al.* determined alcohol content of aqueous solutions and urine by the analysis of the vapour using the static HS approach.³¹ In 1967 the first commercial fully automated HS sampler (Perkin-Elmer Model F-40) was introduced by Perkin-Elmer Instruments³² after Machata successfully introduced the semi-automated headspace sampler.³¹ Today, the complexity of sample matrices coupled with the quest to achieve more accurate and reliable data, have led to the introduction of various types (static, continuous and cryogenic trapping) of headspace sampling techniques. It thus becomes expedient at this point to give a brief overview of the

principles of HS-GC analysis since it was in part used as an analytical tool for investigation of the ladybugs.

Principles of HS-GC Analysis

The underlining principle for HS is the partitioning of the volatile analytes between the vapour and sample phases in the vial. This partitioning can be expressed by the thermodynamically controlled ***equilibrium constant*** (K).³¹ This is synonymous to the partition coefficient as used in gas chromatography,³¹

$$K = \frac{C_s}{C_g} \quad (1)$$

where C_s and C_g are the analyte concentrations in sample and gas phases.

The overall material balance (total mass of analyte in the system, which is the sum of the mass in the sample and the vapour phases) will be given as follows:

$$C_o V_s = C_s V_s + C_g V_g \quad (2)$$

where C_o = original concentration of analyte in sample

V_s = volume of analyte in the sample

V_g = volume of analyte in headspace

Combining equations (1) and (2), the following equation can be derived:

$$C_o V_s = K C_g V_s + C_g V_g \quad (3)$$

From the above equation, C_o can be written as,

$$C_o = C_g \left(K + V_g / V_s \right) \quad (4)$$

The ratio of V_g/V_s (β) is known as the phase ratio and it represents the ratio of the volumes of the two phases present:

$$\beta = \frac{V_g}{V_s}$$

Equation (4) can therefore be written as,

$$C_o = C_g(K + \beta)$$

$$C_g = \frac{C_o}{(K + \beta)}$$

Since K and β are both constants, then equation (7) can now be expressed as

$$C_g = (\text{constant}) \cdot C_o \quad (8)$$

This implies that for a given system, the concentration in the headspace is directly proportional to the original sample concentration.³¹ This follows the basic rule in GC that the peak area for a given analyte is proportional to its concentration in the sample.³¹ Thus, for HS analysis, the peak area, say A , obtained from the GC analysis is proportional to the headspace concentration C_g .³¹

$$A = (\text{constant}) \cdot C_g \quad (9)$$

The equilibrium constant (partition coefficient) is a primary factor that expresses the mass distribution in the two-phase system and is dependant on the solubility of the analyte in the condensed phase.³¹ Analytes that have high solubility in the sample phase relative to the headspace phase ($C_s \gg C_g$) will have high K values and vice versa.³¹ Note that the greater the value for C_g the greater the peak area.

Many, if not most analytical methods involving ultratrace analytes have intrinsic errors, which often lead to unreliable and irreproducible data. In this regard, Allen, *et al.* proposed the stable isotope dilution method as a means of obtaining more accurate results.⁸

The Stable Isotope Dilution Method

The use of an internal standard is crucial for most quantitative analysis since it is practically unattainable to reproducibly introduce exactly the same quantity of sample into the analytical instrument always. Other factors such as losses through sampling and extraction method, evaporation and derivatisation, adsorption unto glassware, possible decomposition particularly in the GC injector and adsorption unto column will influence recovery of analyte(s). In order to control these factors, the choice of internal standard is crucial. The best internal standard must at least possess the same chemical properties as the analyte(s), i.e., be of the same homologue where possible. Therefore, the use of the isotope label of the analyte as an internal standard is widely accepted.

The stable isotope dilution method is an analytical technique in which the stable isotope-labeled analogue of the analyte is used as an internal standard. An advantage of this method is that the isotope-labeled analogue of the analyte will not be present naturally in the sample.

With recent advances in the use of hyphenated analytical systems, the GC/MS provides an effective means for distinguishing between the isotope-labeled analyte

(internal standard) and the analyte itself. Distinct retention times for the isotope-labeled and the analyte can be obtained with the MS scanning for different masses. Qualitative and quantitative determinations are achieved by first identifying the respective peaks and then determining the ratios of the measured peak areas of the analyte and the internal standard in the sample over a relevant concentration range. In the case of this research, the trideuterated analogue of IPMP was used as the internal standard for the analysis of the three 2-alkyl-3-methoxypyrazines in wine samples.

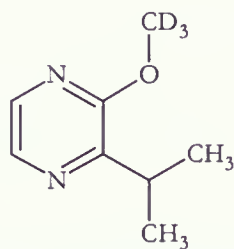


Figure 1.5 Structure of trideuterated 2-isopropyl-3-methoxypyrazine

Molecularly Imprinted Polymers (MIPs)

MIPs are synthetic polymers with well-defined surface cavities (imprints) created by template molecules. The use of MIPs in analytical separation science has become common.^{32,33} MIPs have been applied to several separation techniques, mostly chromatographic-based and solid phase extractions.³⁴ The great interest in MIPs is primarily due to their potential to selectively recognize the analyte (high affinity for the target molecule) and hence the possibility to adsorb it from the bulk sample, which are mainly environmental and biological

systems.³⁴ MIP-based separations are focused on making a shape-selective cavity that contains strategically positioned functional groups, which enables recognition and selective adsorption of the analyte (template). An initial mixing of the template and the monomer(s) gives a pre-polymerized complex in a selected solvent. Subsequently, polymerization is initiated with a cross-linker agent to form solid polymers in the form of beads. The polymer beads are often ground and sieved to obtain the appropriate particle size. The adsorbed template is consequently isolated from the monomer, which leaves cavities of complimentary shape, size and functionality. Following preparation, the MIPs then “recognize” the analyte in the presence of other molecules in solution under appropriate conditions by shape selectivity and binding groups on cavity surface.

Although MIPs are easy to prepare, inexpensive and amenable to various analytical chemistry fields, some current issues of concern include;

- stabilization of monomer–template complexes in the pre-polymerization mixture
- complete removal of template molecules from the polymer formed and
- suitable design of polymer format for intended use.

Statement of the Problem

In the late summer of 2001, Eastern North America including southern ontario experienced a population explosion of ladybugs on the heels of a huge increase in the population of soybean aphids.¹⁹ The *Harmonia axyridis*, unlike other members of the Coccinellidae, have the habit of aggregating in larger numbers as the weather cools down in autumn. As the fall of 2001 advanced, wineries in southern ontario were inundated by huge numbers of these ladybugs, which were inevitably incorporated into the must from which wines were made.²⁰ The incorporation of their defensive secretion imparted to the wines a taste and odour that masked to varying degrees the varietal “bouquet” of the particular grape from which the wine was made. This resulted in various types of wine from a particular grape losing their distinctive taste and odour that distinguish them from others.²⁰ The lack of clear distinction between the wines was attributed to the presence of 2-alkyl-3-methoxypyrazines being imparted to the wine by the ladybugs. In this regard, the major aim of this research work was to determine whether ladybugs especially *Harmonia axyridis* do have any direct association with 2-alkyl-3-methoxypyrazines and hence confirm its possible effect on the odour and taste characteristics of the wine.

The specific objectives used to achieve the major aim were:

- Use GC/MS and headspace coupled with GC/MS to determine the presence and content 2-alkyl-3-methoxypyrazines in various ladybugs (*Harmonia axyridis*, *Coccinella septempunctata*, *Hippodamia convergens*)

- Develop a suitable analytical technique for the extraction and pre-concentration of the 2-alkyl-3-methoxypyrazines from wine
- Use GC/MS for the identification and quantitation of the 2-alkyl-3-methoxypyrazines
- Ascertain the possible incorporation of the 2-alkyl-3-methoxypyrazines by determining the presence and amount of these compounds in ladybug-tainted and commercially available wine samples.
- Employ the use of molecularly imprinted polymers for the removal or reduction of the 2-alkyl-3-methoxypyrazines in wine samples

Due to the fact that the ladybugs are also known to secrete alkaloid toxins, which may tend to incorporate some bitter taste into the wine, this research also set out to investigate the possible association of some alkaloids with *Harmonia axyridis*, *Coccinella septempunctata*, and *Hippodamia convergens*.

CHAPTER 2

Experimental

Headspace – GC/MS Analysis of Ladybugs

Sampling

Three species of ladybugs (*Harmonia axyridis*, *Coccinella septempunctata*, and *Hippodamia convergens*) were obtained from Natural Insect Control (NIC) in Stevensville, Ontario, Canada during the summer. The ladybugs were immediately frozen in order to reduce any further loss of the volatile compounds. Prior to their analyses, the species were allowed to thaw and the average weights of each species determined, by separately weighing 30 ladybugs of each species using an electronic balance (Denver Electrical balance). The ladybugs were then analyzed for 2-alkyl-3-methoxypyrazines using the Perkin-Elmer TurboMatrix 40 headspace sampler.

Similar analyses were performed on another set of the ladybugs using only the *Harmonia axyridis* and *Coccinella septempunctata*. However, these species were obtained during the latter part of the fall season from a volunteer who handpicked them in and around the area of Grimsby, Ontario, Canada. This set of ladybugs was also treated in a similar manner as described earlier.

Instrumental Settings for ladybugs Analysis

A Perkin-Elmer GC (AutoSystem XL) with high purity helium as carrier gas coupled with the TurboMass Gold MS was used for qualitative and quantitative analyses. The instrument, which has a National Institute of Standards and Technology (NIST)

library installed, was used for confirmation of the identified peaks. A DB-5 capillary column (J&W Scientific/Agilent Technologies, USA; 30m x 0.25 μ m x 0.25mm) was used for analyses of the species obtained from NIC while the RTX-5 Sil MS (Rextek Corporation, USA) which is equivalent to a DB5-MS capillary column used for the late fall ladybug species. The initial oven temperature was 40 °C. The temperature was held for 3 min and then ramped to 250 °C at 10 ml min⁻¹, and finally held for another 10 min. The analysis was done with a split flow set at 10:1 and the carrier gas flow at 1.0 ml/min.

The mass spectrometer was set up in the Selected Ion Monitoring (SIM) mode. A total of 13 masses that formed the major peaks found in the mass spectra of the three compounds (IPMP, SBMP and IBMP) were monitored using electron ionization (EI) mode. The MS had a solvent delay of 4 min and the source temperature was set at 150 °C with the GC/MS interface at 250 °C.

With the headspace sampling, the transfer line from the headspace unit to GC was maintained at 95 °C, while the column head pressure was set at 12 psi. Equilibration was achieved at 90 °C for an optimal time of 20 min with the shaker on. Injection time was 0.5 min. Prior to each run, the GC/MS was auto tuned and calibrated to enhance its sensitivity and also ensure the instrument's reliability.

Determination of Headspace Equilibration Time

In order to ensure a more reliable and reproducible sampling technique, the equilibration time for the headspace sampling procedure was determined. The

analysis was carried out by thermostating at different times (5, 10, 15, 20 and 25 min) a known aqueous standard (50 µg/L) containing all the three analytes. The same instrumental settings as described earlier were maintained. The equilibration time for each compound was then determined by obtaining on the same graph, a plot of chromatographic peak area versus time of each compound.

Analytical Procedure

Mixed standards of the three compounds (IPMP, IBMP and SBMP) were used to determine the relative amount of each methoxypyrazine from a stock solution of 1.0 mg/L prepared by weighing appropriate mass of each methoxypyrazine compound into a 1000 ml volumetric flask. Serial dilutions of the stock solution were prepared ranging from (0.5–600) µg/L. Each solution was immediately sealed with Parafilm[®] and kept in the refrigerator. Double distilled water was used as blank. Due to the wide range of concentrations for the aqueous standards (0.5–600 µg/L), different plots [5-point calibration curves; (0.5–10) µg/L, (5.0–100 µg/L) and (50.0–600 µg/L)] for each compound were obtained. This approach was adopted to ensure that area obtained for each methoxypyrazine compound from the ladybug species, fell within the limits of calibration. Plots of each calibration curve were used, when possible, to quantify the three different methoxypyrazines present in each ladybug species.

The stock solution (1.0 mg/L) was prepared by dissolving 1 mg, weighed to ± 0.002 of each standard into a 1.0 L volumetric flask containing double distilled water. The

volumetric flask was covered with aluminium foil to minimize the possibility of photolysis and the mixture was rapidly stirred for 24 h in the dark for equilibration. Approximately 5.0 ml volume of each standard were placed in the headspace vials, *ca.* 23.0 ml and immediately capped. The vials were then placed in the TurboMatrix 40 headspace sampler and thermostatted, as described earlier. Freshly prepared standards were used prior to each analysis. A total of 5 ladybugs of each species type were placed in the headspace vial and also analyzed using the same instrumental settings. All samples were run in duplicates.

From the calibration curves for each standard, the relative amounts of alkylmethoxypyrazines in each ladybug species were determined.

GC/MS Analysis of Ladybugs

Extraction Procedure

The ladybugs were initially macerated and extracted using methylene chloride as the final solvent. Two types of extraction techniques were employed (Figure 2.1).

1. Direct extraction of the ladybugs with methylene chloride
2. Extraction with methylene chloride after previously treating the macerated species with HCl (36.5 % m/v) and NaOH (97.0 % m/v) solutions. The chemicals were all obtained Caledon Laboratories Limited, Georgetown, Ontario)

In the first (1) extraction method, as a result of the direct use of methylene chloride, the coloured pigments of the ladybug species were also extracted. To avoid

contamination and/or overloading of the GC column, the extracts were initially cleaned up using a micro-column packed with silica gel before their final GC/MS analysis. Methanol (20% v/v) in methylene chloride was used as the elution solvent and the separate fractions were each analyzed on the GC/MS. Below is a flow chart showing both methods used for the extraction process.

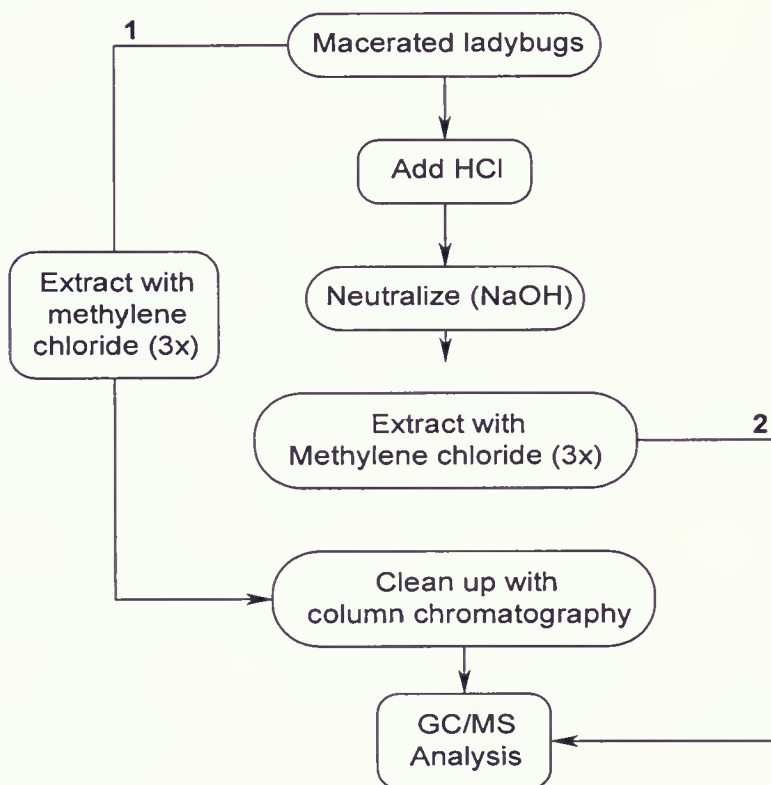


Figure 2.1 Chemical extraction procedures of macerated ladybugs

With the second (2) extraction method, the ladybugs were first treated separately with varying concentrations (0.5, 1.0, and 2.0 M) of HCl solutions. Each fraction was then

neutralized with equivalent concentration of NaOH solution and extracted with 3 portions of methylene chloride as indicated in the flow chart.

GC/MS Analysis of Wine Samples

Two sets of wine samples were analyzed,

- a) Riesling wine samples tainted with ladybugs (*Harmonia axyridis*)
- b) Commercial wine samples of different grape varieties.

The Riesling ladybug-tainted wine samples were obtained from Dr. Gary Pickering of Cool Climate Oenology and Viticulture Institute (CCOVI), at Brock University. The white wine samples tainted with 3-ladybugs/L wine, were prepared in 2003 whereas those tainted with 10-ladybugs/L wine in 2001. A non-tainted white wine sample from the same grape juice extract was used as a control. The tables below provide a summary of the condition and type of some of the wine samples used in the analyses.

Table 2.1 Conditions and types of tainted wine samples

Wine sample	Number of ladybugs/L wine	
	3 ladybugs/L wine	10 ladybugs/L wine
Riesling wine	corked	uncorked
Red wine	n/a	uncorked
Year Prepared	2003	2001

n/a: not available

Commercial wine samples were obtained from a local winery.

Table 2.2 Commercial wine samples obtained from local winery

Symbol	Year	Type
W1	2003	Riesling (Tank sample)
W2	2001	Riesling (Bottled 2002)
W3	2003	Riesling
W4	2001	Vidal
W5	2001	Pinot Blanc
W6	2001	Gewurztraminer
O1	2001	Rose
R1	2001	Baco (Bottled 2002)
R2	2003	Baco

Wine Extraction Procedures

Solvent Extraction Technique

Each wine (tainted red and white wines) sample (150.0 ml) were pipetted separately into clean 250 ml separatory funnel and 5.0 % w/v of NaCl crystals were added. The mixture was stoppered and shaken until the salt dissolved. The mixtures were then spiked with 50 µg/L isotope-labeled IPMP (final concentration in wine is approximately 50 ng/L) and extracted with three separate 50 ml portions of methylene chloride. The organic layers were separated into a round bottom flask, dried with anhydrous Na₂SO₄ (Laboratory Reagent Grade, BDH Inc. Toronto) and finally filtered with a Whatman[®] filter paper (number 4). The methylene chloride extracts were then pre-concentrated to approximately 1.0 ml volume on a rotary evaporator at *ca.* 30 °C. The 1.0 ml final extracts were transferred into GC vials and the volume further reduced to 0.5 ml with argon for GC/MS analysis. A 5 ppb mixed standard prepared in water was also extracted using the same extraction procedure.

Steam Distillation Technique

Each wine sample (150 ml) was measured into a clean 250 ml round bottom flask and 5% w/v NaCl salt was added. The mixture was then spiked with 50 µg/L of isotope-labeled IPMP. The mixture was distilled with steam for 40 min and the distillate collected over ice chips. The collected distillate was pre-concentrated using the Oasis[®] solid phase extraction (SPE) cartridge fitted onto a Supelco Visiprep[®] SPE vacuum manifold. To ensure effective interaction with the cartridge, the vacuum pressure was kept low (1.5 psi) at a reduced flow rate.



Figure 2.2 Supelco Visiprep[®] solid phase extraction vacuum manifold

Prior to the pre-concentration step, the SPE cartridge was prepared by running 2.0 ml each of methanol, 10% methanol in water and double distilled water in that order. This ensures that the reverse phase material of the cartridge is well activated for adsorption of analytes onto its surface. After the pre-concentration step, the cartridge was dried under vacuum. The analytes were then desorbed into vials using 1.0 ml methylene chloride. Further pre-concentration was done by evaporating the solvent with argon to a final volume of 0.5 ml for GC/MS analysis.

Preparation of Stock and Standards

A stock solution was prepared by initially dissolving approximately 100 mg of IPMP, IBMP and SBMP respectively into 1000 ml volumetric flask containing double distilled water. The mixture was stoppered immediately, covered with aluminium foil and stirred for 24 hr for equilibration. A further 100-fold dilution was made to give a final concentration of 1.0 mg/L which served as the stock solution. The solutions were well stoppered and stored in the refrigerator to reduce any possible losses. The standards were prepared by making serial dilutions of the stock solution to give concentrations ranging from 5 – 500 ng/L. Each of the standards was also carried through the same extraction procedure as the wine samples.

Instrumental Settings for GC/MS Analysis of Wine

All GC injections were made in the splitless mode. In the case of the analyses involving solvent extractions, the GC initial temperature was 30 °C and that was held for 3 min. The temperature was increased at a rate of 10 °C/min to 140 °C and then at 45 °C/ min to 220 °C. The temperature was finally held for another 3 min.

All other injections were made at an initial oven temperature of 35 °C. The temperature was held for 3 min and then ramped at rate of 10 °C min⁻¹ to a final temperature of 180 °C, which was held for another 3 min. The injector temperature was maintained at 180 °C with a carrier gas flow at 1 ml/min. Four masses (124, 137, 138, 140) were monitored using the MS in the EI mode with a solvent delay of 11 min. The GC interface was kept at 250 °C and the source temperature at 150 °C.

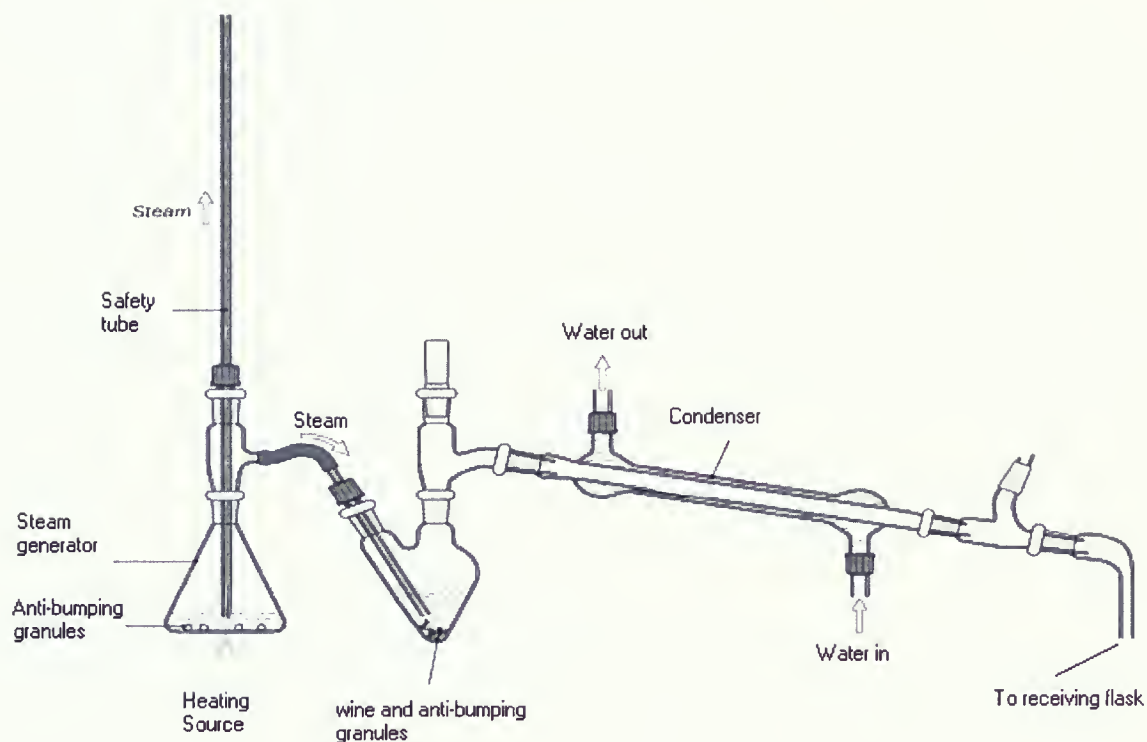


Figure 2.3 Modified Experimental Setup for Steam Distillation Extractions³⁴

Method Validation for Steam Distillation Extraction Technique

Determination of Percent Recovery

Three separate concentrations 0.2, 0.5 and 1.0 $\mu\text{g/L}$ (150 ml each) of the mixed standard were prepared in double distilled water, 12 % v/v ethanol and the wine control sample. Batch extractions of each of these mixtures spiked with the isotope-labeled IPMP were performed using steam distillation followed by pre-concentration on the SPE cartridge. A procedure similar to the one described earlier for the wine samples were used in each case. Each mixture was then analyzed for the three methoxypyrazines by GC/MS. Calibration curves for standards were obtained and the percent recoveries of the methoxypyrazines determined in each case.

Effect of Salting-out technique on Extraction Efficiency

This set of experiments was performed using the tainted white wines obtained from CCOVI. In this case, batch extractions on 5 separate wine samples were performed. Prior to the steam distillation extraction, 0, 5, 10, 15, 20% w/v NaCl were dissolved in each wine sample. The same extraction procedure and analyses as described earlier were used. The total area of each methoxypyrazine peak was determined and compared.

Analyses of Plastic Barrel Wine Samples

A 2002 Riesling white wine sample kept in a sealed plastic barrel was obtained from Dr. Gary Pickering, CCOVI. Three separate extractions were done using the same steam distillation–SPE extraction method and isotope dilution technique. Mixed standards of concentrations ranging from 5 to 50 ng/L of the three methoxypyrazines were also extracted using the same procedure. The amount of methoxypyrazines in each extract was determined from a calibration plot for each compound. The standard deviation and precision were then determined.

Analyses of wine samples after treatment with MIPs

Already prepared MIPs with IPMP as the template and methacrylic acid as the functional monomer were used in this set of experiments. The cross linking agent used was ethylene glycol dimethacrylate from Sigma Aldrich, Oakville, Ontario, Canada. The particle sizes of the IPMP imprinted polymers were generally less than

10 μm . Non-imprinted polymers (NIPs) were also prepared using a similar method but without the template. Dr. Lu Chunyang with the Chemistry Department, Brock University, prepared both MIPs and NIPs used for these experiments.

Wine samples (50 ml each) were measured into Erlenmeyer flasks and 100 mg of the MIPs and NIPs were separately added. The mixtures were immediately sealed with a Teflon[®] cork and shaken on a mechanical shaker for 20 hr at 120 rpm. Later the mixtures were filtered into 100 ml round bottom flasks and the filtrates (wine samples) were steam distilled. The distillate was collected over ice chips and extracted with three portions of 25 ml methylene chloride. The organic layer was separated, dried with anhydrous Na_2SO_4 and then pre-concentrated to 1 ml on the rotary evaporator under ambient conditions. The final extract was analyzed with similar GC/MS procedure as described earlier. Sensory analyses were also performed²³ by a team of experts from CCOVI (Brock University) led by Dr. Gary Pickering using the same MIPs-treated wine samples. A similar process as described was repeated using only the wine sample without the addition of MIPs and NIPs.

CHAPTER 3

RESULTS AND DISCUSSION

Headspace – GC/MS Analysis of Ladybugs

Data analyses for the two replicates were limited to the range of calibration for each particular compound's sensitivity (Appendix A1-A4). That is, although each calibration curve was used to quantify each alkylmethoxypyrazine, only values that fall within the limit of calibration for a particular curve were chosen. The amounts of the three methoxypyrazines were obtained from the average peak area of each of the duplicates. Thus, the amount for each analyte was expressed as mass per mg and also as mass per ladybug for each species based on the average peak area for each duplicate (Tables 3.1 – 3.4). An elaboration of the data, indicating the peak areas with the corresponding standard deviation, is found in the appendix A4.

Table 3.1 Average amounts ($\mu\text{g mg}^{-1}$) of three methoxypyrazines in the ladybug species obtained in late summer (n=2)

Compound	Ladybug Species		
	<i>Harmonia axyridis</i>	<i>Hippodamia convergens</i>	<i>Coccinella septempunctata</i>
IPMP	0.8	5.7	< 0.1
SBMP	0.1	0.4	nd
IBMP	0.1	1.1	nd

In the case of late summer ladybugs (Table 3.1), *Harmonia axyridis* was found to contain nearly equal amounts of SBMP and IBMP. However, the relative amount of IPMP was higher in all three ladybugs. In *Coccinella septempunctata*, SBMP and IBMP were not detected.

Table 3.2 Average amounts (μg per ladybug) of the three methoxypyrazines in the ladybug species obtained in late summer (n=2)

Compound	Ladybug Species		
	<i>Harmonia axyridis</i>	<i>Hippodamia convergens</i>	<i>Coccinella septempunctata</i>
IPMP	27.5	90.8	0.4
SBMP	2.6	5.6	nd
IBMP	3.2	17.3	nd

All three species were found to contain IPMP with *Hippodamia convergens* having relatively higher amount than the other two species examined. SBMP and IBMP were present in both *Harmonia axyridis* and *Hippodamia convergens* with SBMP found to be the methoxypyrazine compound with the lowest concentration.

From the results, it is evident that if *Hippodamia convergens* had aggregating behavior similar to *Harmonia axyridis*, it would pose the highest threat to wine production due to high levels of methoxypyrazines found in them. The relatively lower concentrations for SBMP corroborate reports of their moderately lower concentrations found in some wines.^{7, 24}

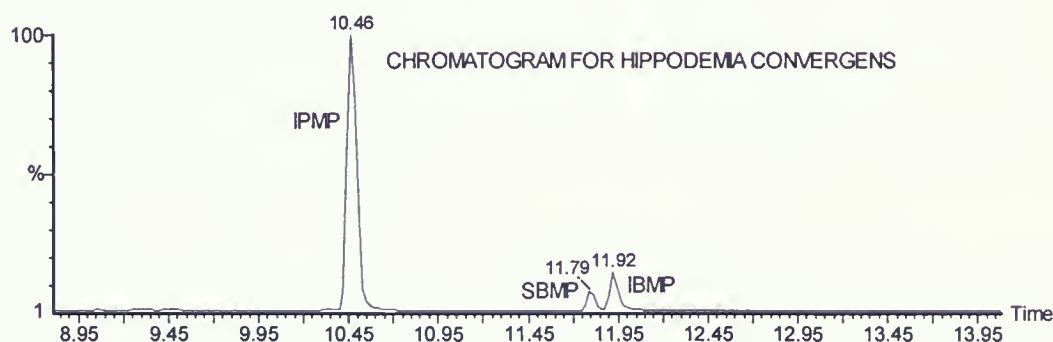


Figure 3.1 TIC Chromatogram of *Hippodamia convergens* from HSA

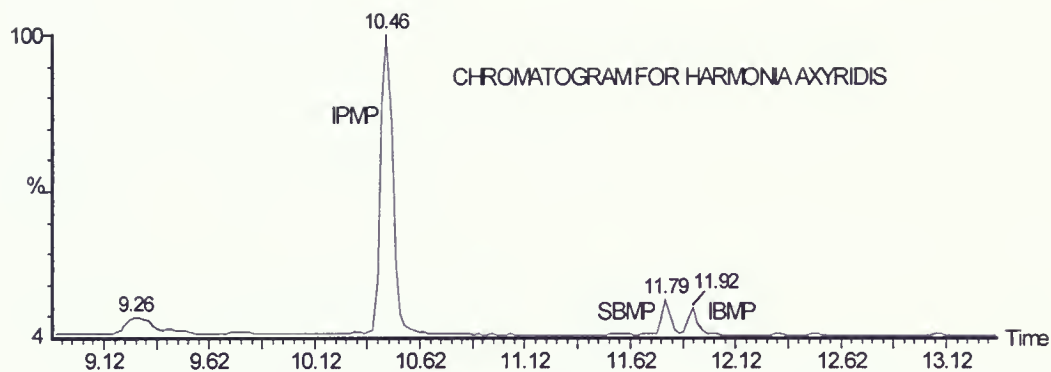


Figure 3.2 TIC Chromatogram of *Harmonia axyridis* from HSA

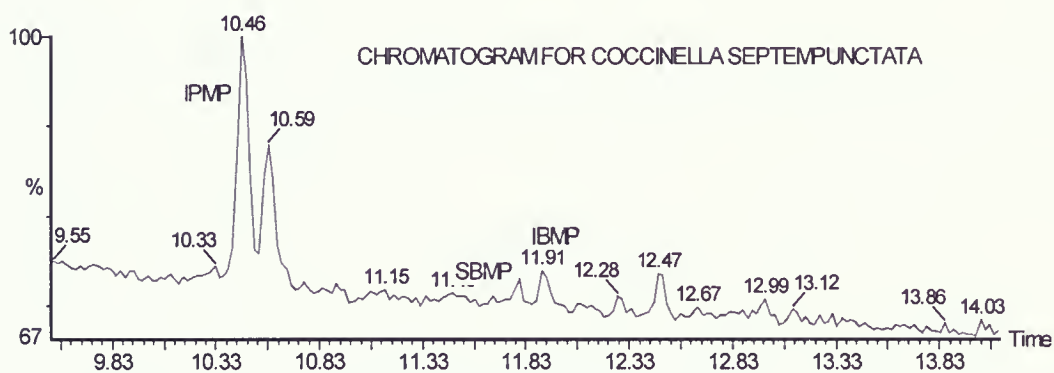


Figure 3.3 TIC Chromatogram of *Coccinella septempunctata* from HSA

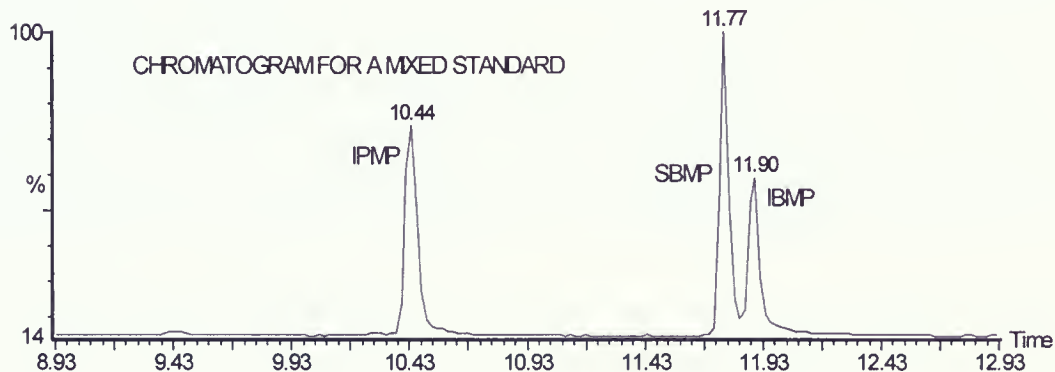


Figure 3.4 TIC Chromatogram of a mixed methoxy pyrazine standard for HSA*

* HSA – Headspace Analyses

Although the characteristic vegetal aroma is often attributed to the presence of IBMP, results suggest that IPMP will likely have the greatest effect due to its higher concentrations in the three ladybug species studied.

The average weight of *Coccinella septempunctata* was recorded as 50 mg. *Harmonia axyridis* gave 34 mg while *Hippodamia convergens* was 16 mg.

In agreement with the standards (Figure 3.4), the retention time obtained for IPMP was 10.46 min, followed by 11.79 min for SBMP while IBMP gave a retention time of 11.92 min for the ladybugs.

For the late fall headspace analyses of *Harmonia axyridis* and *Coccinella septempunctata*, results show relatively higher amounts for IPMP than SBMP and IBMP. Same amounts of methoxypyrazines were recorded for SBMP and IBMP (Table 3.3 and 3.4).

Table 3.3 Average amounts ($\mu\text{g}/\text{mg}$) of three methoxypyrazines in the ladybug species obtained in late fall

Compound	Ladybug Species			
	<i>Harmonia axyridis</i> [1]	<i>Harmonia axyridis</i> [2]	<i>Coccinella septempunctata</i> [1]	<i>Coccinella septempunctata</i> [2]
IPMP	0.8	0.03	< 0.01	0.02
SBMP	0.1	0.01	nd	0.01
IBMP	0.1	0.01	nd	0.01

[1] Results for ladybug species obtained in late summer [2] Results for ladybug species obtained in late fall

Compared to analyses of the late summer species, the late fall ladybugs showed a similar trend for the amounts of all three methoxypyrazines. In contrast, the *Harmonia axyridis* species gave relatively lower amounts of methoxypyrazines while *Coccinella septempunctata* recorded higher levels.

Table 3.4 Average amounts (μg per ladybug) of the three methoxypyrazines in the ladybug species obtained in late fall

Compound	Ladybug Species			
	<i>Harmonia axyridis</i> [1]	<i>Harmonia axyridis</i> [2]*	<i>Coccinella septempunctata</i> [1]	<i>Coccinella septempunctata</i> [2]*
IPMP	27.5	1.1	0.4	1.2
SBMP	2.6	0.4	nd	0.2
IBMP	3.2	0.4	nd	0.2

[1] Results for ladybug species obtained in late summer [2] Results for ladybug species obtained in late fall

The ladybug species obtained in the late summer were from NIC, Stevensville under well packaged (perforated sealed plastic containers) and controlled manner. The late fall ladybugs were obtained from a volunteer who sampled the species in and around the Grimsby area in Ontario.

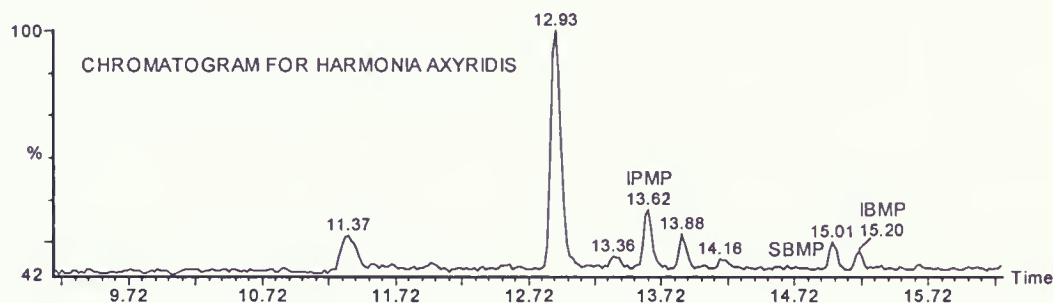


Figure 3.5 TIC Chromatogram of *Harmonia axyridis* from HSA

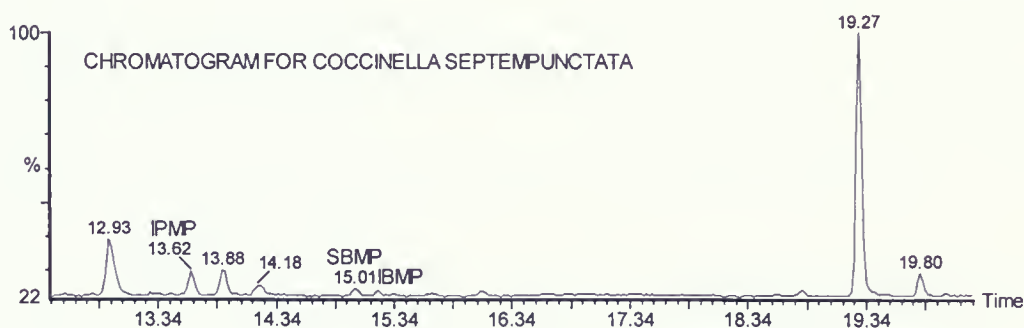


Figure 3.6 TIC Chromatogram of *Coccinella septempunctata* from HSA

The disparity in the results may be attributed to the differences in handling (late fall species hand-picked from the environment into a plastic container over a period of time), sampling technique and source of the ladybugs. Hence, this may have led to the significant losses of methoxypyrazines at varying degrees from the various species. This assertion may be attributed to the fact that the ladybugs whenever disturbed or attacked secrete methoxypyrazine semiochemicals as olfactory alerting signals.¹⁸ By and large, the ladybugs species aggregate in fall at the onset of the colder season and therefore become less prone to disturbances or attacks. As a result, it is expected that if the ladybug species were carefully sampled in the late fall with little or no disturbance, higher levels of methoxypyrazines may be recorded.

From the late fall headspace analyses a distinguishing feature between the two species is the huge peak observed at 19.27 min for *Coccinella septempunctata* (Figure 3.6). A search from the NIST library suggested that the peak was due to a long chain aliphatic alkane (nonacosane, pentacosane, etc). Although *Harmonia axyridis* gave a relatively higher peak at 12.93 min, a similar peak was also observed for the other species. Both species, in addition to showing the peak eluting at 12.93 min, also showed compounds eluting at 13.88 min and nearly 14.20 min. The compounds eluting at these retention times also gave similar results (substituted aromatic compounds) from the NIST library. Some of these compounds included 1,2,3-trimethyl benzene, 3-amino benzoic acid, 6-nitro-3-picoline among others.

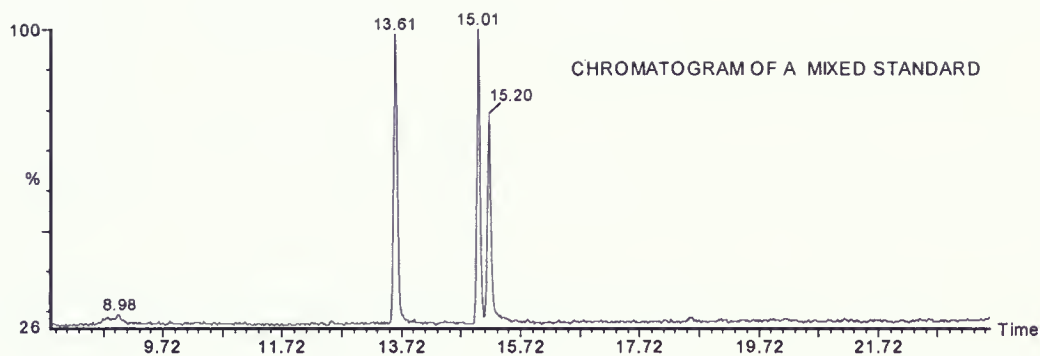


Figure 3.7 TIC Chromatogram of mixed methoxypyrazine standard for HSA

Although it is rare to find insects secreting nitro-containing compounds, the mass spectra of the various matches obtained from the NIST library did not show any close comparable mass spectra apart from the last compound. Thus, the last compound was indicated based on how its mass spectra matches with those obtained from the NIST library.

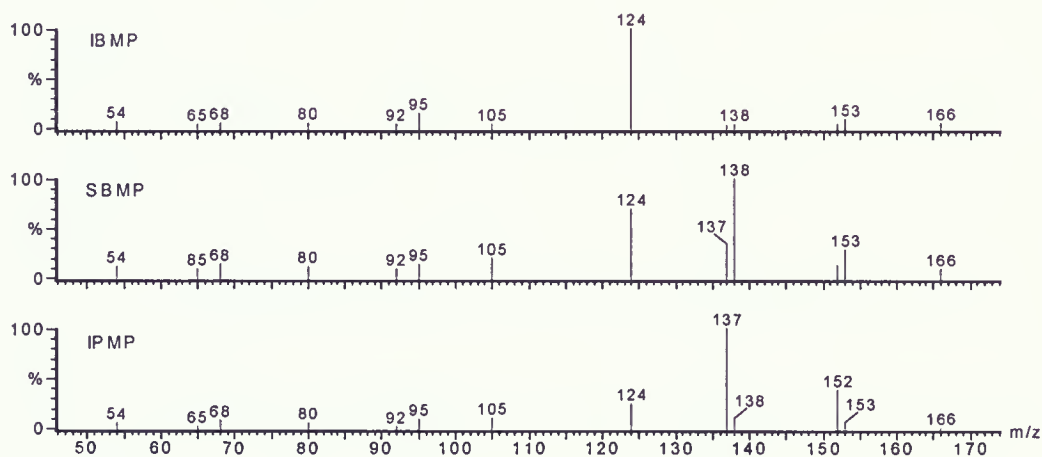


Figure 3.8 Mass spectra for selected masses of IPMP, SBMP and IBMP

The differences in the retention times recorded for each compound from both analyses are due to the slight modification of the column surface material. Although a DB5 30 meter column was used in both cases, in the latter analyses, an RTX-5 Sil MS (equivalent to DB5-MS) with the same specifications was used. This type of column

is designed to be more compatible (significantly lower column bleed) with the mass spectrometer.

From the mass spectra (Figure 3.8), the base peaks for IPMP, SBMP and IBMP are 137, 138 and 124 respectively. The molecular ions for these compounds are 152 for IPMP and 166 for SBMP and IBMP since they are isomeric compounds.

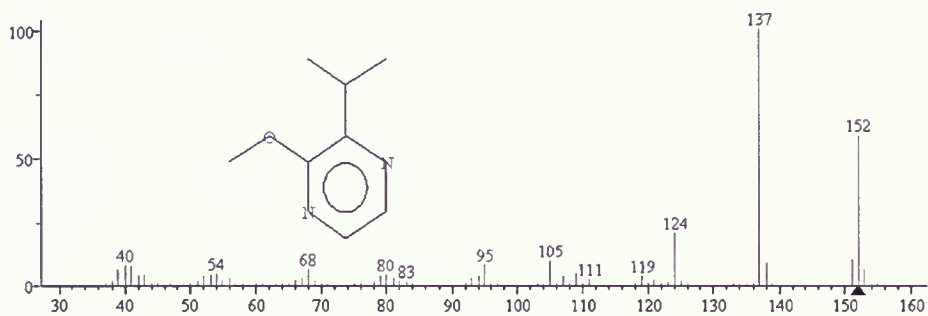


Figure 3.9 Mass spectra of 2-isopropyl-3-methoxypyrazine

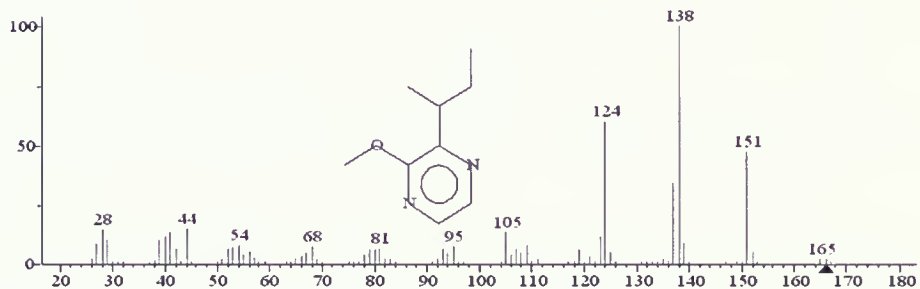


Figure 3.10 Mass spectra of 2-secbutyl-3-methoxypyrazine

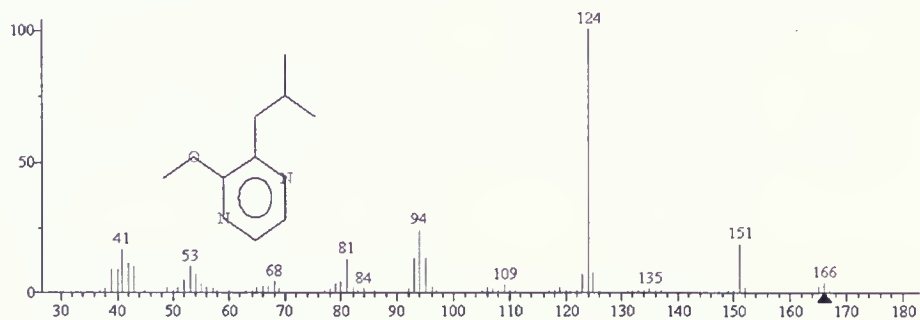


Figure 3.11 Mass spectra of 2-isobutyl-3-methoxypyrazine

Determination of Headspace Equilibration time

The optimized equilibration time of 20 min ensures a good equilibration of the alkylmethoxypyrazines into the gas phase. Although there may be some variation in headspace concentrations of the pyrazines for the ladybugs that might be attributed to the different masses of the ladybugs, this factor should be a minor one.

From Figure 3.12, it can be observed that equilibration is achieved after 15 min of thermostating time. However, the 20 min equilibration time was chosen since it allows ample time for a more reproducible and reliable data.

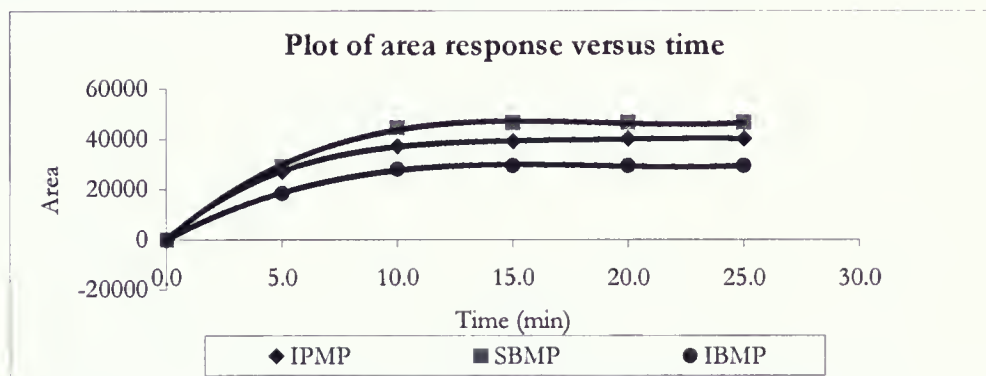


Figure 3.12 Determination of equilibration time for headspace analyses

These data suggest that, if all three ladybugs had similar behaviours, the amount of methoxypyrazines found in the wine would depend on the type of ladybug species in addition to other factors known.³⁰ Fortunately, as noted above, only *Harmonia* exhibits the aggregation behaviour to the degree that this could be a problem.

GC/MS Analysis of Ladybugs

Results obtained for extraction of ladybugs with methylene chloride did not show any peaks for alkylmethoxypyrazines for each of the three ladybug species. However, a huge peak eluting at a retention time of 9.09 min was observed for two of the ladybugs (*Hippodamia convergens* and *Coccinella septempunctata*). This peak gave the corresponding mass spectra (Figure 3.13) with a 192 base peak. The identified target turned out to be the precoccinelline alkaloid. This confirms results obtained elsewhere ¹⁷ for the presence of the coccinelline and the precoccinelline alkaloids in *Coccinella septempunctata*.

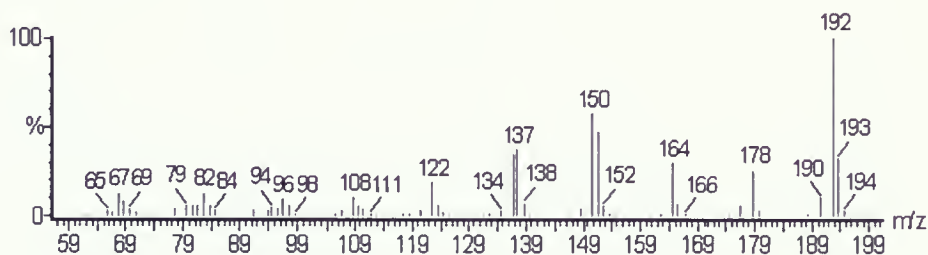


Figure 3.13 Mass spectra of the precoccinelline alkaloid isolated from *Coccinella septempunctata* and *Hippodamia Convergens*

The precoccinelline has the same mass (192) for base peak and molecular ion.

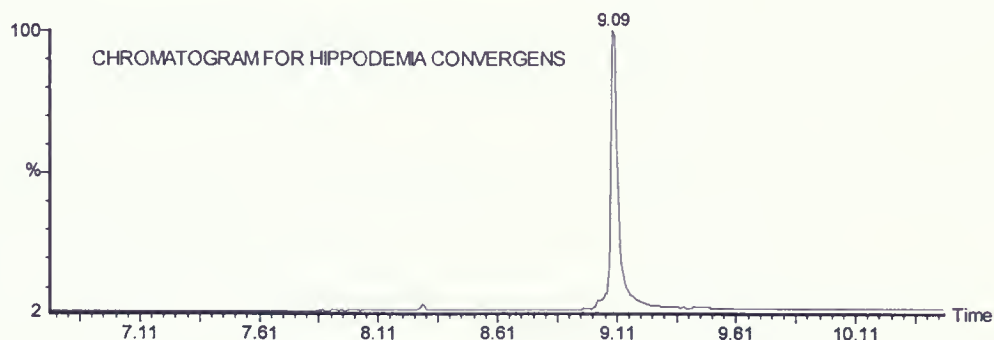


Figure 3.14 Chromatogram of methylene chloride extract of *Hippodamia convergens*

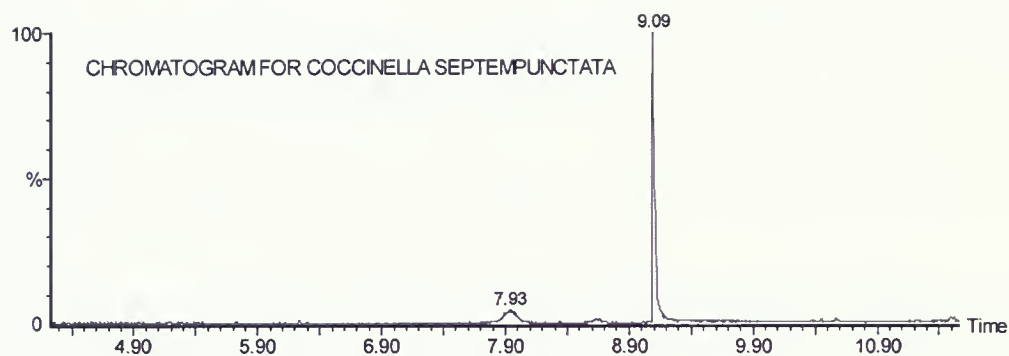


Figure 3.15 Chromatogram of methylene chloride extract of *Coccinella septempunctata*

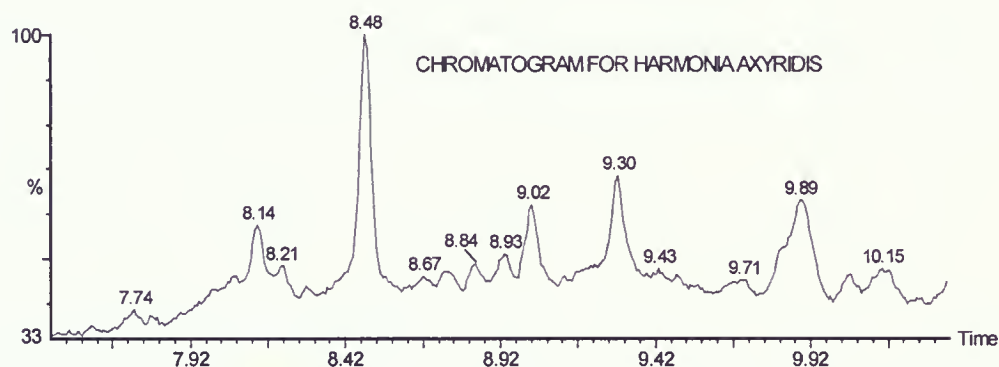


Figure 3.16 Chromatogram of methylene chloride extract of *Harmonia axyridis*

From the data, unknown compounds eluted at various retention times (3.21, 3.56, 4.95, 9.74, 10.44, 10.88, 12.40, 12.60 min) for the various fractions of the methylene chloride extracts (Appendix C1). Using their mass spectra, a search through the NIST library gave no alkaloids but rather long chain substituted aliphatic alkanes and aromatic substituted compounds.

A similar search was done for other eluting compounds from the HCl extracts. This also gave similar results (long chain alkanes and substituted aromatic compounds), although different compounds were observed (Appendix C2). An initial direct

extraction in which hexane was used in place of methylene chloride also did not give any alkaloids nor methoxypyrazines. This may be attributed to the low extraction capacity of hexane due to its non-polar nature.

Although quantitation of this alkaloid was not performed, since the standard is not available, it appears from the total ion count a considerable quantity of this compound is extracted from the ladybug species. This implies that if *Coccinella septempunctata* as well as *Hippodamia convergens* demonstrate similar aggregating activities like the *Harmonia axyridis* species, the likelihood of the wine quality being affected is high. This assertion is due principally to the known bitterness or unpleasantness that is associated with alkaloids.

GC/MS Analysis of Wine Samples

Analyses of Riesling wine samples tainted with ladybugs

Solvent Extraction Technique

Analyses done using solvent extraction technique followed by rotary evaporation gave inconsistent data. Various GC analyses of the same wine sample extracts using identical method produced poorly resolved peak shapes (Figure 3.17). For each of the extracts analyzed by GC, the total peak area of the same analyte in extract gave varying results (Table 3.5). The poor data produced may be attributed to variations in the amount of volatile methoxypyrazines that were lost as a result of the rotary evaporation or from the extraction process.

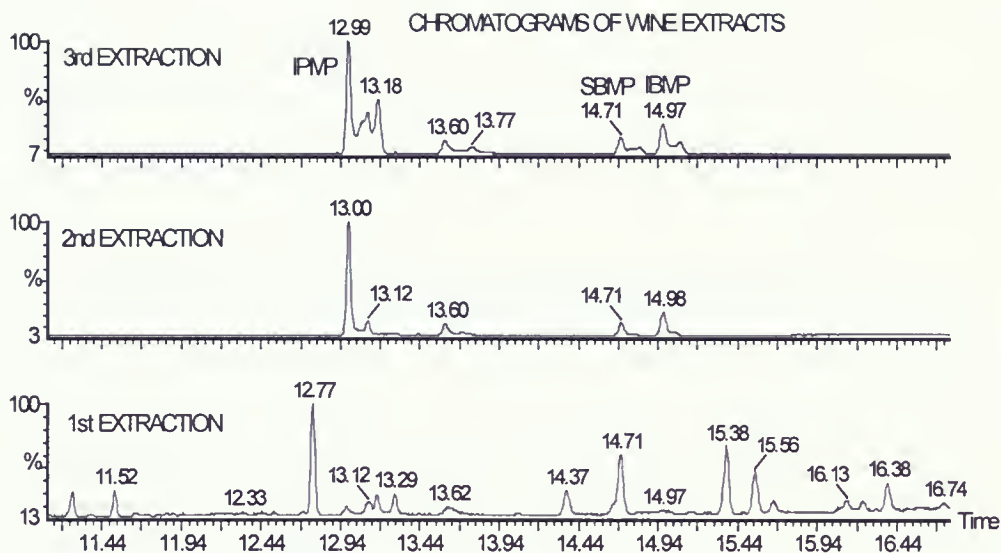


Figure 3.17 Chromatograms of 3 ladybug/L wine extracts using solvent extraction

Despite the fact that three separate 50 ml portions of methylene chloride were used for the extractions, the possibility of losing some of the analytes through transfer from one glassware to another, and by means of analytes adhering unto the glassware perhaps contributed to the inconsistencies observed from the data.

Table 3.5 Results obtained for the extraction of Riesling wine tainted with 3 ladybugs using solvent extraction technique

Compound	Peak Areas for three replicates		
	[1]	[2]	[3]
IPMP	560	6060	3200
SBMP	3340	850	530
IBMP	280	1600	1040
Standard Deviation: IPMP (2750) SBMP (1540) IBMP (670)			

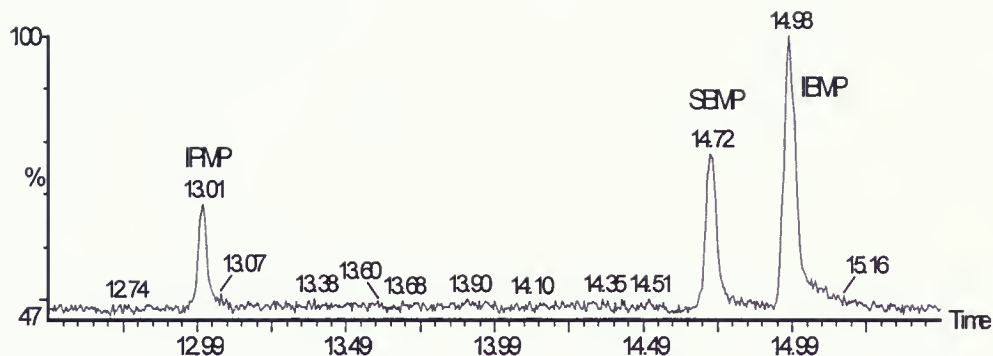


Figure 3.18 Chromatogram of a 5 ppb mixed standard of methoxypyrazines using solvent extraction method

From the chromatogram obtained for an extract of a 5 ppb mixed standard (Figure 3.18) using the same solvent extraction technique, IPMP, SBMP and IBMP elute at 13.01, 14.72 and 14.98 min respectively. Comparing the chromatogram for the standard and that for the wine extracts, it is evident that the solvent extraction method may be more suitable for relatively higher analyte concentrations (parts per billion range) because, at very low concentrations (parts per trillion), as is the case for the methoxypyrazines in wine extracts, any possible losses of analytes contribute to significant changes in the data obtained.

Steam Distillation Extraction Technique

Comparing the data for the two different wines, results (Table 3.6) indicate higher amounts of methoxypyrazines in samples tainted with 3 ladybugs/L than those with 10 ladybugs/L of wine. This difference might be due to storage method and/or wine age. Though, the latter wine samples had higher levels of ladybugs and thus, expected to contain higher levels of methoxypyrazines, ²⁰ those samples were stored in

uncorked (sealed with a Teflon™ screw-cap) bottles. This must have led to relatively higher losses of some of the volatile methoxypyrazines compared to those samples (3 ladybugs/L wine samples) stored in corked bottles. In addition, the uncorked samples (10 ladybugs/L wine samples) were also prepared in 2001 compared to the former, which were prepared in 2003. Consequently, the additional storage time may lead to some higher losses of the volatile methoxypyrazines through evaporation and possibly wine aging. ²⁴

Table 3.6 Results obtained for Riesling white wines tainted with ladybugs

Compound	Concentration (ng/L) (n = 1)	
	(A)	(B)
IPMP	29.4	8.5
IBMP	7.6	<5.0
SBMP	<5.0	nd
nd: not detectable	(A): 3 ladybugs/L wine	(B): 10 ladybugs/L wine

Higher amounts of IPMP were detected than their corresponding methoxypyrazines in both wine samples. SBMP was the lowest amount of methoxypyrazine present in both wine samples, with sample **A** recording < 5.0 ng/L whereas in sample **B** SBMP was not within detectable limits.

From the chromatogram (Figure 3.19), it can be noticed that IPMP, SBMP and IBMP elute at 12.41, 13.67 and 13.98 min respectively whereas the isotope labeled IPMP (deuterated IPMP) elutes at 12.37 min. Comparing the chromatograms for wine samples **A** and **B**, the latter (Figure 3.21) showed no peaks for SBMP and IBMP while that of IPMP was fairly well-defined. The wine sample tainted with 3

ladybugs/L of wine (sample **A**), on the contrary gave more distinct peaks for all of the three analytes (Figure 3.21). This observation generally confirms the lower concentrations of methoxypyrazines recorded for wine samples tainted with the 10 ladybugs/L of wine. This is because from the chromatographic principles the peak area is directly proportional to the concentration of analyte. Thus higher analyte concentrations result in greater peak areas.

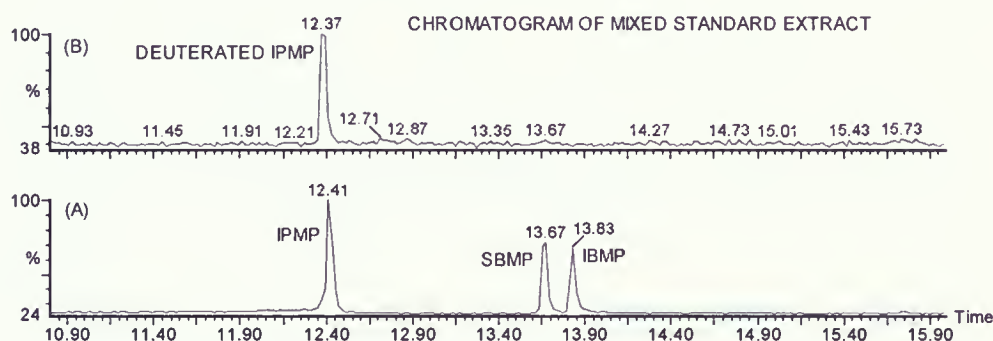


Figure 3.19 Chromatogram of mixed standard using steam distillation
 (A). TIC Chromatogram of mixed standard (B). Mass Chromatogram of deuterated IPMP

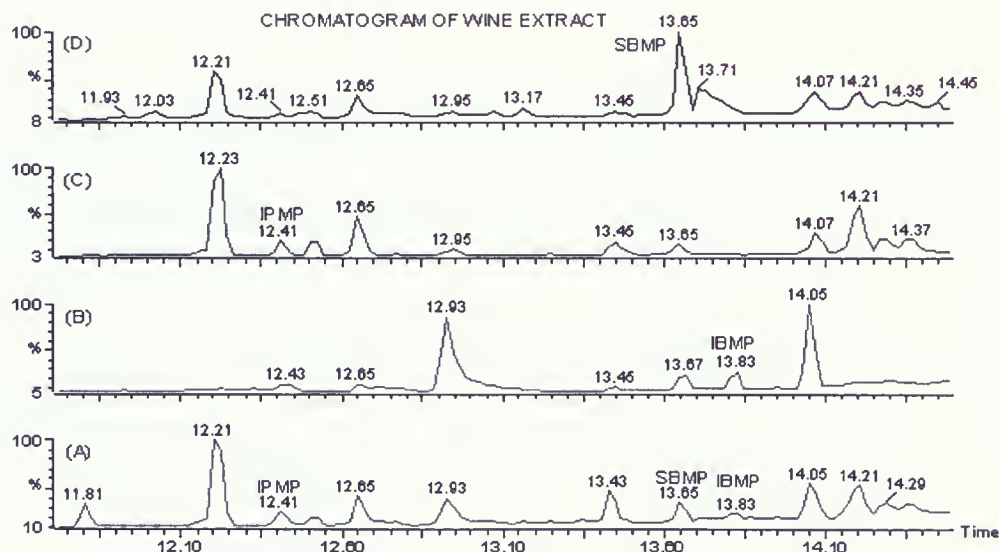


Figure 3.20 Chromatogram of wine extract tainted with 3 ladybugs
 (A). TIC Chromatogram of wine extract (B). Mass Chromatogram of IBMP
 (C). Mass Chromatogram of IPMP (D). Mass Chromatogram of SBMP

Analyses of commercial wine samples of different grape varieties

For a total of 9 different commercial wine samples analyzed, concentrations of methoxypyrazines within detectable limits, ranged from a low concentration of 6 ng/L to 260 ± 10 ng/L (Table 3.7).

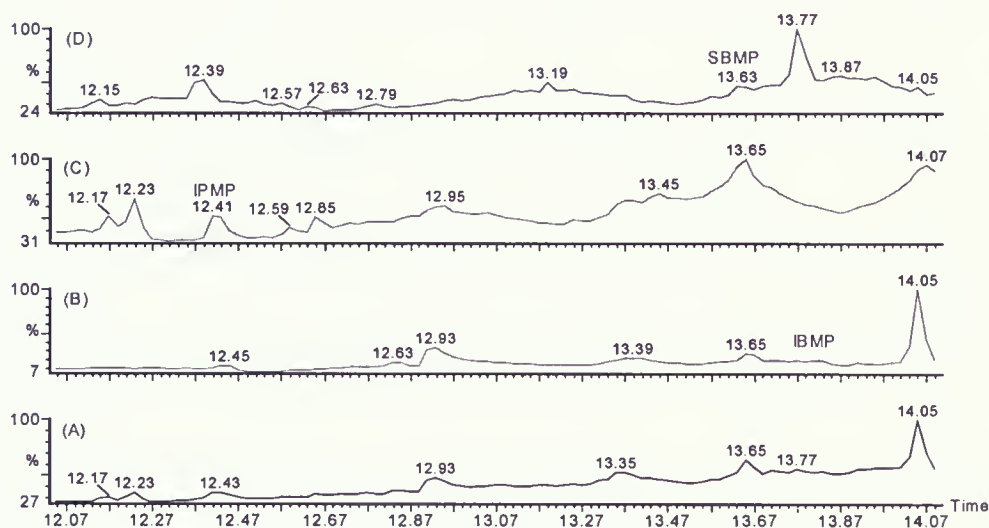


Figure 3.21 Chromatogram of wine extract tainted with 10 ladybugs

(A). TIC Chromatogram of wine extract

(B). Mass Chromatogram of IBMP

(C). Mass Chromatogram of IPMP

(D). Mass Chromatogram of SBMP

Table 3.7 Levels of methoxypyrazines found in some commercial wine samples

Sample	Year	Concentration of methoxypyrazines (ng/L) (n=2)			
		IPMP	SBMP	IBMP	TOTAL
W1	2003 Riesling (tank sample)	87*	37*	45*	169
W2	2001 Riesling (bottled 2002)	113*	56*	102*	271
W3	2003 Riesling	163 ± 3	114 ± 5	127 ± 4	404
W4	2001 Vidal	260 ± 10	107 ± 5	119 ± 7	486
W5	2001 Pinot Blanc	192 ± 5	113 ± 3	245 ± 9	550
W6	2001 Gewurztraminer	175 ± 4	nd	244 ± 4	419
O1	2001 Rose	159*	61*	72*	292
R1	2001 Baco (bottled 2002)	nd	nd	nd	-
R2	2003 Baco	38*	6*	21*	65
AVERAGE TOTAL		148	71	122	

W: white wine sample R: red wine sample

O: orange appearing wine

*limited sample

With the exception of wine samples W5 and W6, IPMP was the highest concentration of methoxypyrazine. Incidentally, IBMP was the highest concentration of methoxypyrazine in these wine samples. The wine samples generally were found to contain relatively lesser amounts of SBMP compared to IPMP and IBMP. None of the three methoxypyrazines were detected for wine sample R1 whereas in sample W6, SBMP was also not detected.

Two different year (2001 and 2003) batches of wine samples were analyzed. From the analyses, it was observed that the 2001 wine samples recorded relatively higher concentrations of methoxypyrazines than the 2003 samples with the exception of R1 sample, in which no analyte was detected (Table 3.7). It is important to note that the above statement is made based on the total number of samples (3 and 6 samples for 2003 and 2001 respectively) analyzed in each case. The failure to detect any of the analytes in R1 samples is attributable to the lower levels of methoxypyrazines generally found in red wines. In addition, wine sample R1 was initially prepared in 2001 and bottled in 2002. Thus most of the volatile methoxypyrazines may have been lost through evaporation as a result of poor storage. Another possibility is that the grapes might have been harvested at a date when no ladybugs were aggregating in the vineyards. This is because the Baco grapes are normally harvested earlier before the aggregation of the ladybug species. Wine sample W2 which was also stored in a similar manner to R1, however contained fairly significant levels of methoxypyrazines. This could be due to rather unusual high levels of methoxypyrazines in this particular white wine sample; hence despite the possibility

of losses through evaporation, significant levels still remained in the wine. With respect to the 2003 wine samples, W3 was found to contain the highest amounts of methoxypyrazines while R2 recorded the least (Figure 3.22).

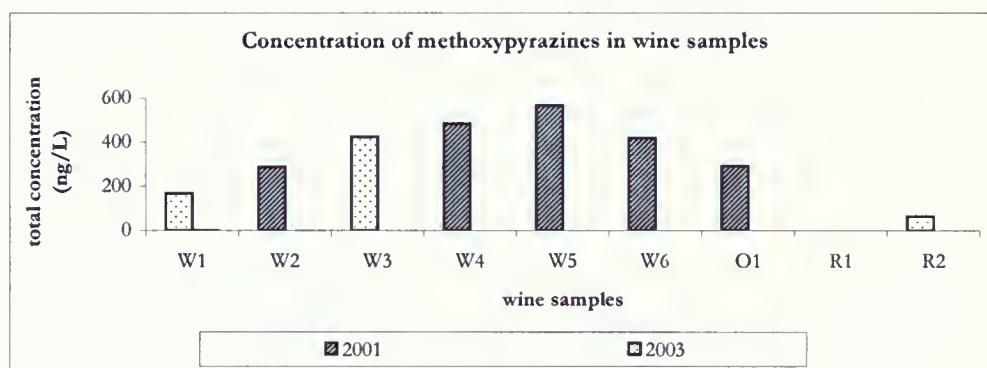


Figure 3.22 Variations of total amount of methoxypyrazines in wine

Correlation between analyses of ladybugs and the commercial wine samples

Results obtained from the headspace analyses of methoxypyrazines in ladybugs indicated higher concentrations for IPMP with nearly equal amounts of IBMP and SBMP (Tables 3.3 and 3.4). A similar trend was observed for IPMP in the wine samples analyzed except for W5 and W6 samples. In terms of IBMP and SBMP, four wine samples (W1, W3, W4 and O1) gave a similar trend to that observed for the ladybugs.

In the case of the analyses of alkylmethoxypyrazines in both ladybugs and wine samples, the following general observations were made from the results:

- Ratios of the overall average amount of each methoxypyrazine in both ladybugs and wine samples were in the order IPMP > IBMP > SBMP (Figure 3.23)

- Concentrations of IPMP in wine samples and ladybugs were higher than IBMP and SBMP
- Amount of alkylmethoxypyrazines contained in 2001 samples were higher than the 2003 wines
- Wines of varying grape varieties contained methoxypyrazines

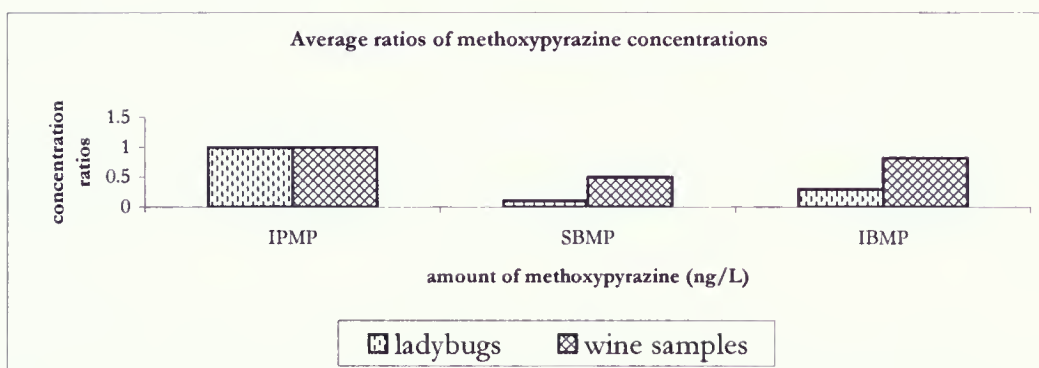


Figure 3.23 Average ratios of methoxypyrazine concentrations in ladybugs and wine samples

The general trends in the levels of methoxypyrazines in both wines and ladybugs may suggest the possible influence of the ladybugs on the amounts of alkylmethoxypyrazines found in the wine samples. In addition, the higher amounts of methoxypyrazines contained in 2001 wine samples support the fact that invasion of the ladybugs on several vineyards in that year may have led to the incorporation of these compounds into wines of various grape varieties. As a result, alkylmethoxypyrazines masked to varying degrees the distinctive aroma/flavour of wines made from a particular grape variety. Elsewhere, Pickering *et al.* observed that an increase in the number (10 ladybugs/L wine) of *Harmonia axyridis* had significant impact on the sensory properties of both red and white wines.²³ The report further

indicated that *Harmonia axyridis* increased the vegetative and herbaceous flavours significantly in white wines and therefore concluded that there is a direct correlation of the vegetative aroma with methoxypyrazines in wines.³⁵ Although, the headspace analyses did not show *Harmonia axyridis* to have the highest amount of methoxypyrazines (Figure 3.24), its potential of having a major impact on the vegetative and herbaceous aromas/flavours in the wines cannot be overruled, especially considering the higher levels of IPMP in both this ladybug species and the wines. This statement is made based on the fact that in 2001 aggregation of ladybugs showed considerably higher numbers of the *Harmonia axyridis* compared to other ladybug species.

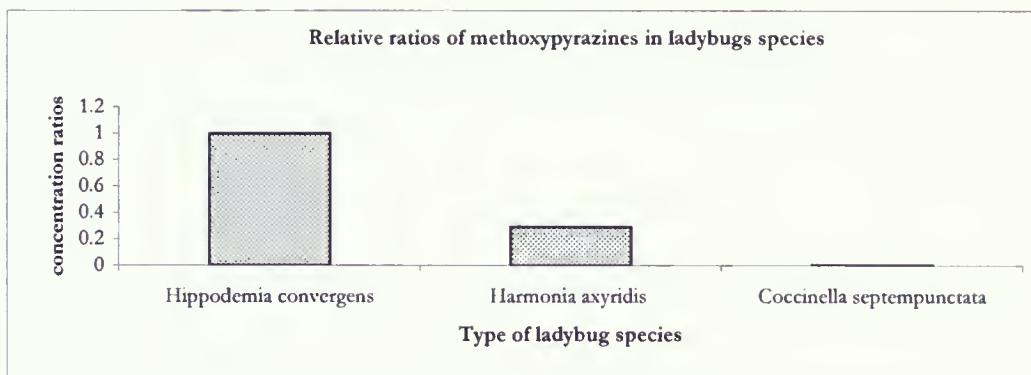


Figure 3.24 Relative ratios of methoxypyrazines in ladybug species

However, in line with the primary objective of this research work, the above indications may be adequately conclusive to make the *Harmonia axyridis* exclusively responsible for the alkylmethoxypyrazines or the herbaceous/vegetative contaminations in wines. This is because while a 10:1 ratio of IPMP to SBMP was found in the *Harmonia* species, a 2:1 ratio was observed for the wine samples.

Similarly, ratios of IPMP to IBMP in *Harmonia axyridis* and the wine samples were not comparable. Allen *et al.* also indicated that high levels of IBMP occur in wines when the grape juice gets into contact with the skin during fermentation process.³⁶ In addition, studies have shown that general occurrence of alkylmethoxypyrazines has a direct relation with the grape variety and vine conditions.³⁶ Other factors like ripening temperature and light exposure of the fruits also influence methoxypyrazine levels in grapes. In this regard, the rather higher levels of SBMP found in the wine samples than in the ladybugs suggest that other factors apart from the *Harmonia axyridis* may influence the methoxypyrazine contaminations in the wines.

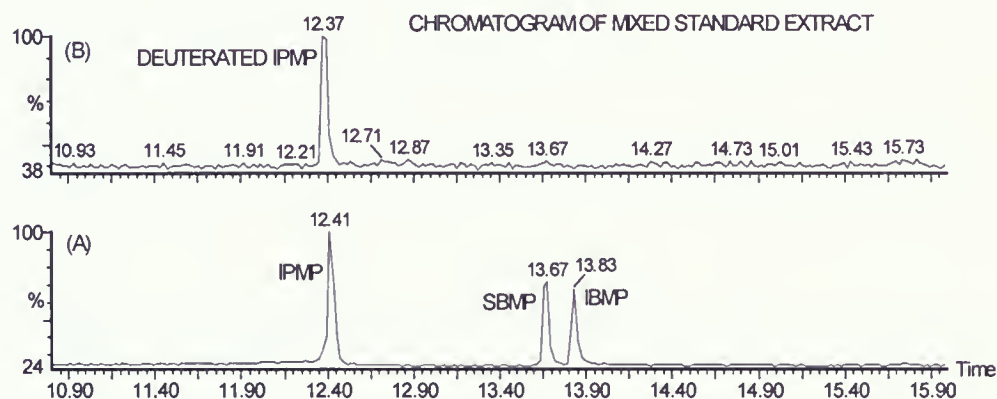


Figure 3.25 Chromatogram of methoxypyrazine standard for commercial wine analyses

From the GC analyses of commercial wine samples, the retention times for IPMP, SBMP and IBMP are 12.41, 13.67 and 13.83 min respectively while the isotope labeled IPMP eluted at 12.37 min (Figure 3.25). With the wine sample extracts, typical retention time for IPMP was 12.46 min while SBMP and IBMP eluted at 13.70 and 13.88 min respectively (Figure 3.26). Figure 3.27 shows the mass spectra for selected masses of each analyte.

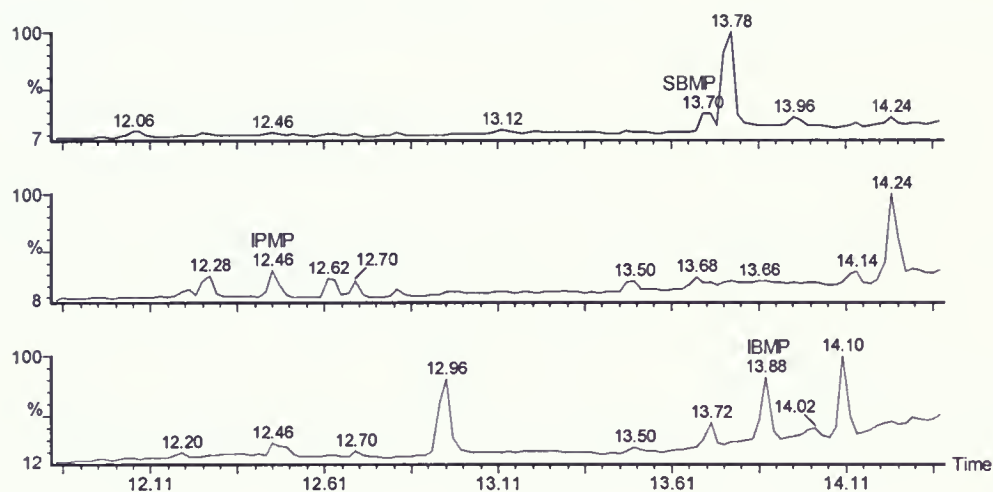


Figure 3.26 Typical mass chromatogram for commercial wine sample extract

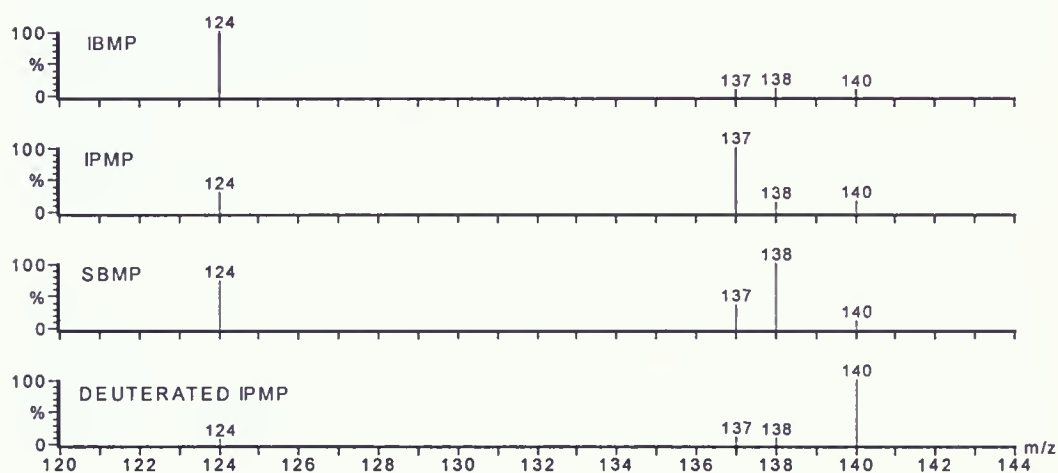


Figure 3.27 Typical mass spectra for selected masses of IPMP, SBMP and IBMP

Compared to the 13 masses (Figure 3.8) used for the HSA of the ladybugs, the four masses in this case were chosen to enhance sensitivity and thus detection. The four masses correspond to the base peaks of each methoxypyrazine including the internal standard. Although different numbers of masses were used in each study, the

difference will not have much difference on the general observation made from both analyses.

Method Validation for Steam Distillation Extraction Technique

Determination of Percent Recovery

The percent recoveries were calculated using a standard calibration curve to determine the amount of each methoxypyrazine in the various spiked samples (control wine, ~12% ethanol and double distilled water) and unspiked control wine sample.

Table 3.8 Amounts of methoxypyrazines recovered for steam distillation (%)

Compound	Sample	Percent Recovery (%) (n=1)		
		0.2 µg/L	0.5 µg/L	1.0 µg/L
IPMP	Wine	84	91	99
	Ethanol	86	93	99
	Water	87	93	99
IBMP	Wine	87	94	99
	Ethanol	89	95	99
	Water	89	96	98
SBMP	Wine	85	95	96
	Ethanol	87	96	98
	Water	89	97	99

The amount recovered from each extract was then calculated using the formula below.

$$\% \text{ recovery} = \frac{\text{total amount in spiked wine extract} - \text{total amount in unspiked wine extract}}{\text{total amount spiked}}$$

Results largely indicate high recoveries for the steam distillation extraction method.

The general observation is that there is an increase in the amount recovered for each

analyte as the concentration of the analyte increases (Table 3.8). This is because an increase in concentration implies that the total amount of each analyte in solution is increased. Thus, more of the analyte can easily be extracted such that minor losses do not have major effect on the overall amount of methoxypyrazine obtained.

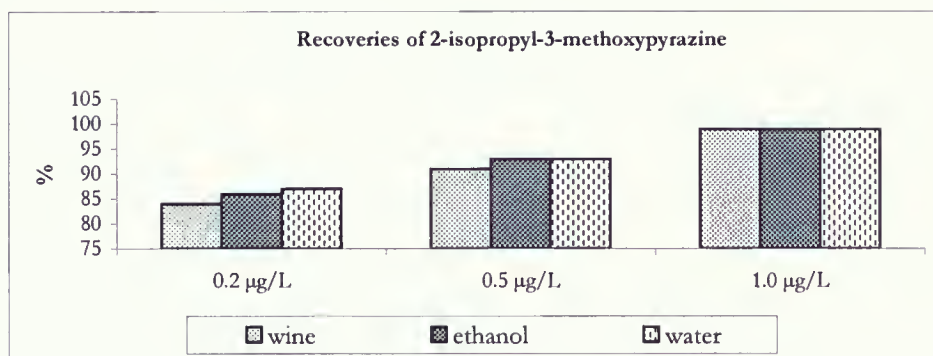


Figure 3.28 Percent recovery of IPMP at three different concentrations

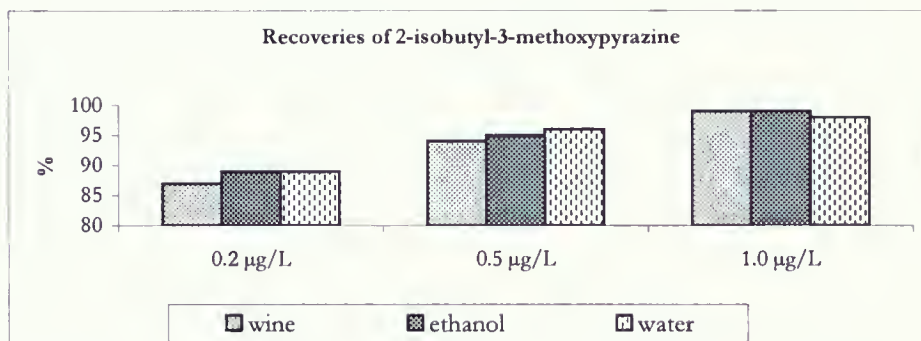


Figure 3.29 Percent recovery of IBMP at three different concentrations

Comparing the extraction efficiencies from the wine, ethanol and water matrices, there is slight increase in the amount of each analyte extracted (Figures 3.28, 3.29 and 3.30) as the matrix changes from wine to water. This observation is attributed to the effect of the complexity of the matrix on the extraction efficiency.

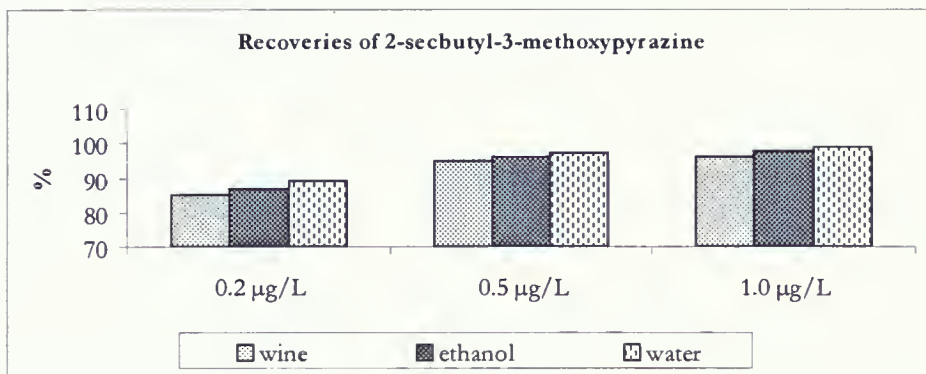


Figure 3.30 Percent recovery of SBMP at three different concentrations

Thus, analytes in more complex matrices will have lower recoveries and vice versa. The wine matrix being more complicated recorded relatively lower recoveries compared to the ethanol and water matrices. However, the matrix effect tends to decrease at higher concentrations (1.0 µg/L). Comparing amount of each methoxypyrazine recovered from each matrix, it was again observed that the percent recovery for each analyte increase with concentration increase.

From the results, it can also be noticed that nearly equal amounts of each methoxypyrazine were extracted for each particular matrix concentration. That is, for a particular solution matrix, the amounts of IPMP, SBMP and IBMP extracted at a specific solution concentration were nearly constant. This implies that steam distillation offered a very effective extraction method while the deuterated labeled IPMP (isotope dilution), as internal standard was also very effective in the quantitation of each methoxypyrazine.

Effect of Salting-out technique on Extraction Efficiency

Results showed that dissolution of 5% (m/v) NaCl into the wine prior to the steam distillation extraction had a remarkable effect on the amount of analyte extracted (Figure 3.31). It was observed that addition of 5% (m/v) NaCl had nearly the same effect as the higher concentrations of the same salt. This effect suggests that a minimal amount of 5% (m/v) NaCl was sufficient to transfer nearly all the analyte into the gaseous phase during the steam distillation process. Though an increase in the concentration of NaCl from 5 to 20% (m/v) increases the total ionic strength, it does not have any effect on the overall amount of analyte extracted.

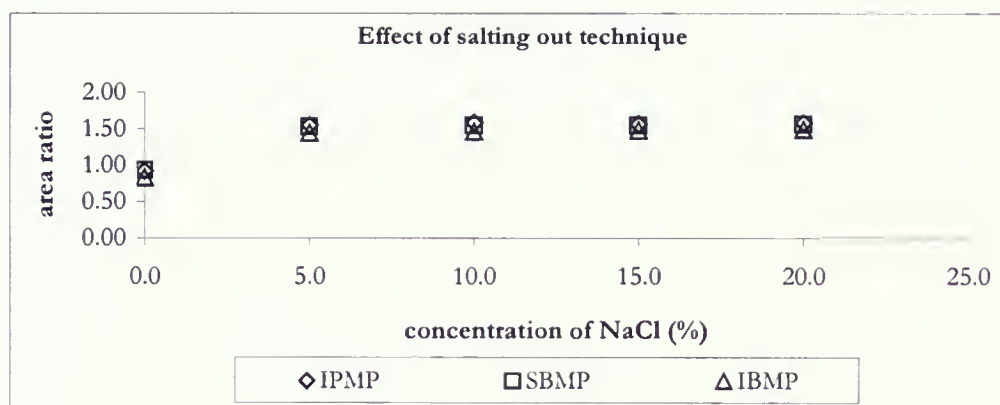


Figure 3.31 Effect of salting out technique on extraction efficiency

This observation may be attributed to the fact that, with the steam distillation method, analytes are distilled at a temperature substantially below that of the boiling point(s) of the individual constituent(s). Hence, the addition of higher concentrations of NaCl did not have any major effect, as the steam under atmospheric conditions will easily carry the analytes into the vapour phase.

Analysis of Plastic Barrel Wine Samples

Table 3.9 shows results obtained for the analyses of 2002 Riesling wine sample obtained in a plastic barrel. From the results IPMP was determined to be 29 ± 1 ng/L while IBMP was estimated to be <5 ng/L. Although a minor peak was detected for SBMP, integration of this peak was not possible (Figure 3.32) because of the relatively lower concentration of the SBMP in the wine sample.

Table 3.9 Analyses of methoxypyrazines in barrel stored wine

Compound	Concentration (ng/L)				Standard Deviation (n=3)
	Replicate 1	Replicate 2	Replicate 3	Average	
IPMP	27.6	28.8	29.6	29	1.0
IBMP	<5	<5	<5	nd	nd
SBMP	nd	nd	nd	nd	nd

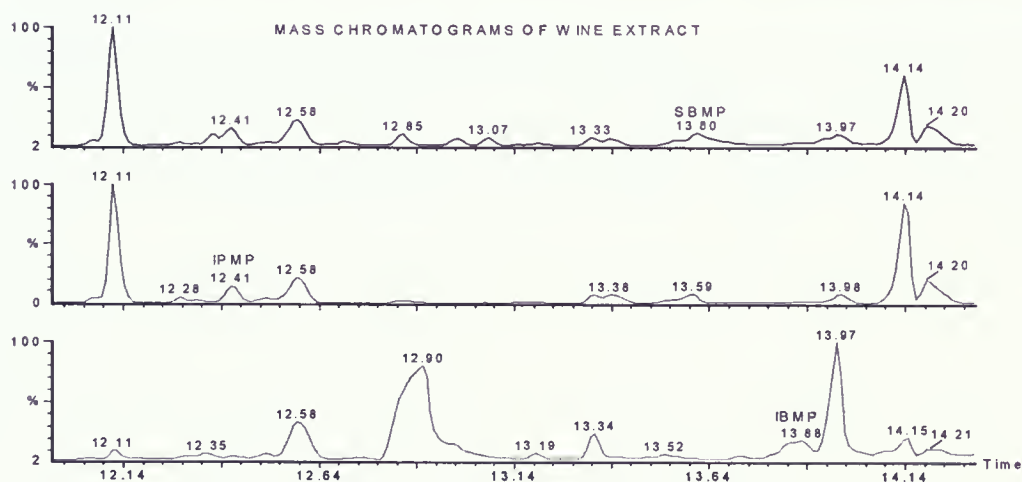


Figure 3.32 Mass chromatograms for Riesling wine sample stored in plastic barrel

The results gave a standard deviation of 1.0 for three extractions which implies that, compared to the solvent extraction method, by and large the steam distillation method coupled with SPE pre-concentration method offers more reliable and

reproducible data, and thus provides an accurate alternative for the determination of methoxypyrazines in wine sample.

Analyses of wine samples after treatment with MIPs

Results as shown in Table 3.11 indicate a general decrease in the total ion count (TIC) or peak area of each analyte after treatment of wine samples with the MIPs. This implies a consequent decrease in the amount of the alkylmethoxypyrazines after treatment of the wine samples with the MIPs. The decrease in the levels of the alkylmethoxypyrazine contaminants is due to their interaction(s) with the MIPs possibly through adsorption onto the imprints.

Table 3.10 Result for the analyses of MIPs treated and non-treated wine samples

Compound	PEAK AREA						Percent Adsorb		
	Non-treated Samples			MIPs Treated Samples			(%)		
	[1]	[2]	[3]	[1]	[2]	[3]	[1]	[2]	[3]
IPMP	745	763	1004	247	534	760	67	30	24
SBMP	160	183	163	nd	94	84	nd	49	48
IBMP	229	299	210	119	197	122	38	34	42
nd: not detected MIPs Stdev: IPMP (510±10); SBMP (90±10); IBMP (150±40) n=3									

From the results, it is obvious that the MIPs show varying adsorption capacities for the alkylmethoxypyrazines. In the case of the IPMP, different amounts were adsorbed by the MIPs for each trial. On the other hand, although there was some variation, adsorption for SBMP and IBMP by the MIPs tended to be fairly constant. This general trend can be due to the fact that the isotope dilution method was not incorporated in the extraction technique. Thus the errors inherent in the extraction method could not be eliminated to any large extent.

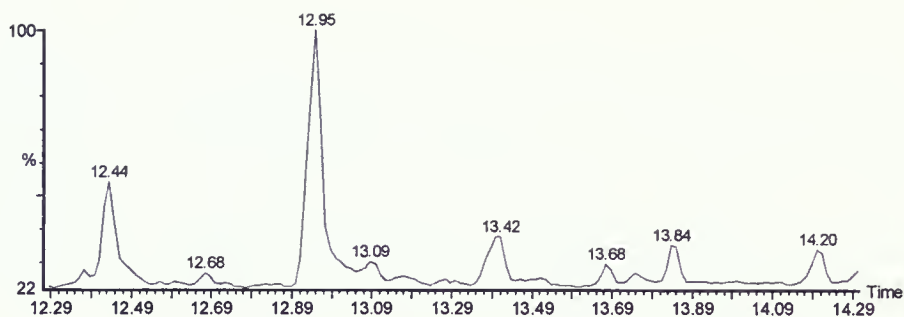


Figure 3.33 Chromatogram of wine sample before MIP treatment

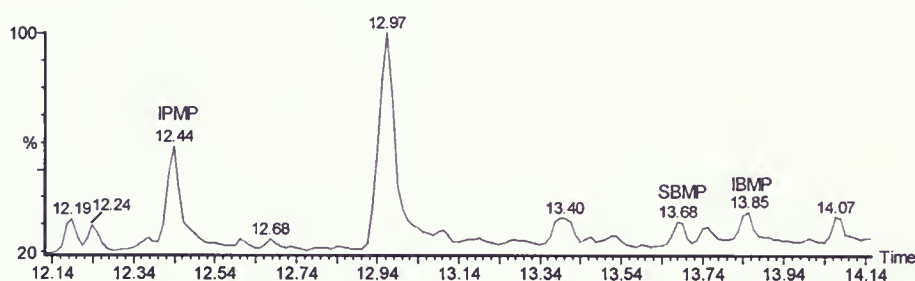


Figure 3.34 Chromatogram of wine sample after MIP treatment

Figures 3.33 and 3.34 show TIC chromatograms for the MIPs treated and the non-treated wine samples. Retention times observed were very reproducible with IPMP, SBMP and IBMP eluting at 12.44, 13.68 and 13.85 min respectively.

Table 3.11a Analyses of MIPs, NIPs treated and non-treated wine samples

Compound	Peak Area								
	Non-treated Samples			MIPs Treated Samples			NIPs Treated Samples		
	[1]	[2]	[3]	[1]	[2]	[3]	[1]	[2]	[3]
IPMP	691	780	229	143	260	165	191	258	134
SBMP	nd	88	57	nd	69	49	nd	74	35
IBMP	203	674	93	119	197	43	82	258	70
nd: not detected MIPs Sdev: IPMP (190±60): SBMP (60±10): IBMP (120±80) n=3									

Table 3.11 b Analyses of MIPs, NIPs treated and non- treated wine samples

Compound	Percent Adsorbed (n=3)							
	MIPs Treated Samples				NIPs Treated Samples			
	[1]	[2]	[3]	Mean	[1]	[2]	[3]	Mean
IPMP	79	67	28	58	72	67	41	60
SBMP	nd	22	14	18	nd	16	39	18
IBMP	41	71	54	55	60	62	25	49
nd: not detected	MIPs Stdev: IPMP (510±10): SBMP (90±10): IBMP (150±40)							

From Tables 3.11 (a and b), it can be observed that the MIPs and NIPs tend to show similar trends in their adsorption capacity for alkylmethoxypyrazines. Generally, both polymers showed higher adsorption for IPMP followed by IBMP and then SBMP. This observation may be attributed to the relative amounts of the methoxypyrazines present in the wines. The amount of SBMP, compared to the other two methoxypyrazines, is low which suggests that there will be greater tendency for other competing compounds in the wine at relatively higher levels to be adsorbed by the MIPs/NIPs than the SBMP whereas in the case of the IPMP and IBMP, their relatively higher concentration ensures that they are preferentially adsorbed by the MIPs/NIPs.

Results obtained from sensory analyses of wine samples suggest, in apparent contrast with the GC/MS data, an increase in the levels of the alkylmethoxypyrazine contaminants and associated flavours (Figure 3.35). This observation may be attributed to the fact that the MIPs were not purely selective (in terms of shape and binding of analytes) in the removal of the alkylmethoxypyrazines from the wines but that they exhibited a general adsorption of organic compounds. This observation is

justified by the fact that NIPs also showed similar adsorption properties compared to the MIPs.

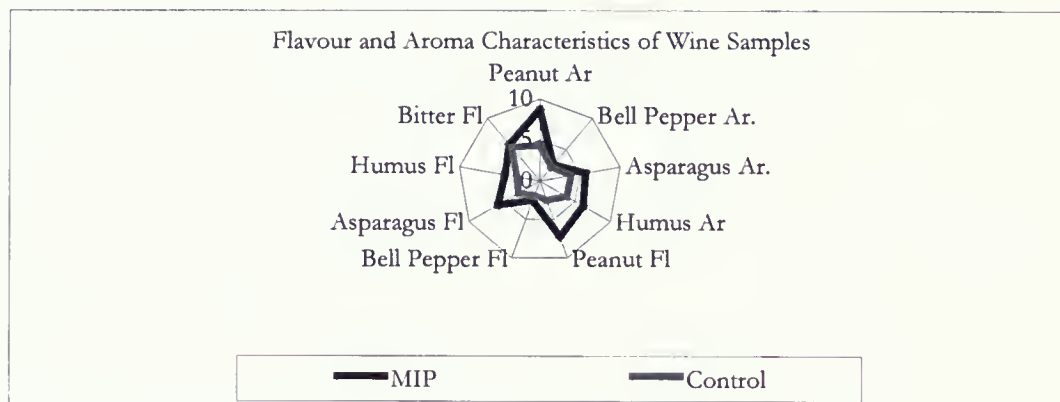


Figure 3.35 Flavour and aroma characteristics of wine samples

With these potential masking volatiles removed and yet the concentrations of alkylmethoxypyrazines in the wine were still within sensory detection, the relative impact of the alkylmethoxypyrazines became greater. Thus, the “masking” effect of other non-methoxypyrazine aroma/flavour constituents was diminished (Table 3.12 and 3.13).

Table 3.12 Results for aroma analyses of MIPs treated and non-treated wine samples

	Peanut Aroma	Bell Pepper Aroma	Asparagus Aroma	Humus Aroma
MIP	8.76	2.61	5.69	6.14
Control	4.35	2.27	3.93	3.8

Control: Non-treated wine sample MIP: MIPs treated wine sample

Table 3.13 Flavour analyses of MIPs treated and non-treated wine samples

	Peanut Flavour	Bell Pepper Flavour	Asparagus Flavour	Humus Flavour	Bitter Flavour
MIP	7.27	2.53	5.93	4.34	5.81
Control	2.51	1.97	2.96	2.85	5.4

Control: Non-treated wine sample MIP: MIPs treated wine sample

CHAPTER 4

CONCLUSION

Headspace coupled with the GC/MS proved successful for the analyses alkylmethoxypyrazines in the ladybugs (Coleoptera: Coccinellidae). It is obvious that the amounts of alkylmethoxypyrazines found in *Hippodamia convergens* indicate that the species may pose potential threat to the wine industry. Fortunately, the *Hippodamia convergens* does not show aggregating behaviour similar to the *Harmonia* species. Hence this possible threat may be less disturbing to the wine industry in view of the fact that fewer numbers of the *Hippodamia* species can be found in ladybug cluster.

The use of the isotope labeled IPMP as internal standard afforded accurate quantitative analysis of the alkylmethoxypyrazines at ultratrace levels (ng/L) in the wine samples. In addition, steam distillation coupled with the SPE pre-concentration technique also offered a successful alternative approach for the determination of alkylmethoxypyrazines in wines.

Headspace and GC/MS analyses of ladybugs and wine samples respectively revealed that the ladybugs may not be the primary cause of the overall effect of alkylmethoxypyrazine contaminations in the wine samples and that other factors which are not made certain at this stage may also some influence. However, trends of IPMP in all the ladybugs and the wine samples, coupled with the extraordinary aggregating behaviour of the *Harmonia* species, suggest that the ladybugs especially *Harmonia axyridis* was the primary source of the high IPMP contaminations in the wine samples.

Despite the effectiveness of the MIPs in reducing the amount of alkylmethoxypyrazines in wines, it did not the best alternative way in removing these compounds from the wine. This is due to the fact that other “masking” organic components for the alkylmethoxypyrazines were also equally adsorbed to the surface of the MIPs.

Recommendations

1. Analyses of alkylmethoxypyrazines in wine samples must be extended to various wineries so as to establish a possible trend of the levels of 2-alkyl-3-methoxypyrazines in both wine and ladybugs species, since this study was focused on a single winery.
2. Increased number of wine samples and monitor the variations in the 2-alkyl-3-methoxypyrazines in wines produced from 2001 till present.
3. Monitor seasonal variations of the alkylmethoxypyrazines in ladybugs species sampled from the environment.
4. Last but not the least, it will be expedient to research into discovering alternative measures/approaches by which levels of the 2-alkyl-3-methoxypyrazines can be reduced significantly, if not eliminated from the wine, without compromising on its quality.

In addition to the above recommendations, another approach that can be used to establish a correlation levels of alkylmethoxypyrazines in wines and ladybugs is by

analyzing a set of ladybug species and wine samples fermented with the same kind of ladybug species.

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Appendix A1 – A4:

Calibration curves for HS-GC/MS analyses and results of late summer ladybugs species

Appendix B1 – B2:

Calibration curves for HS-GC/MS analyses and results of late fall ladybugs species

Appendix C1 – C2:

Chromatograms of wine extracts using solvent extraction method

Appendix D1 – D6:

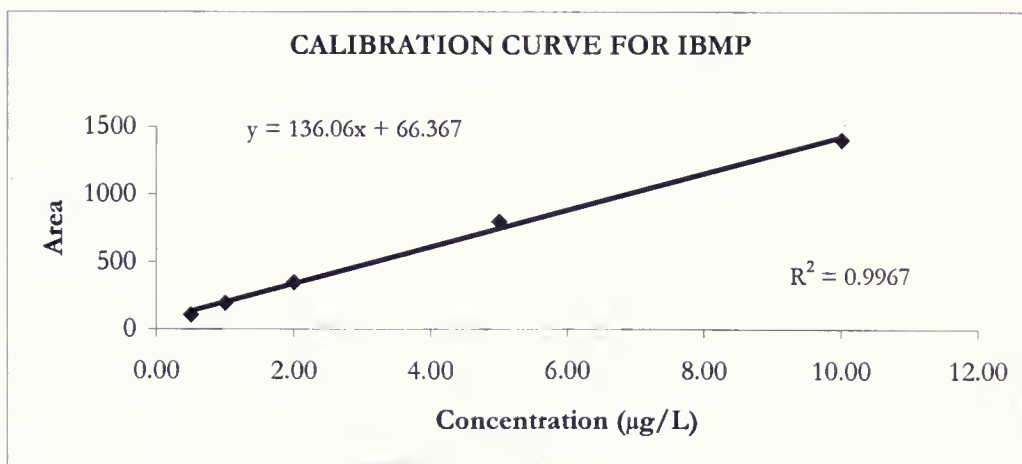
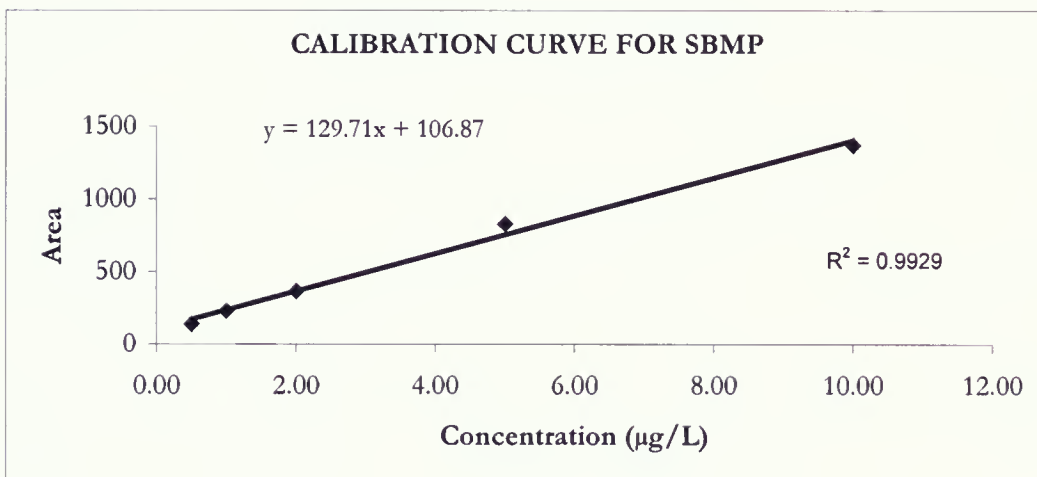
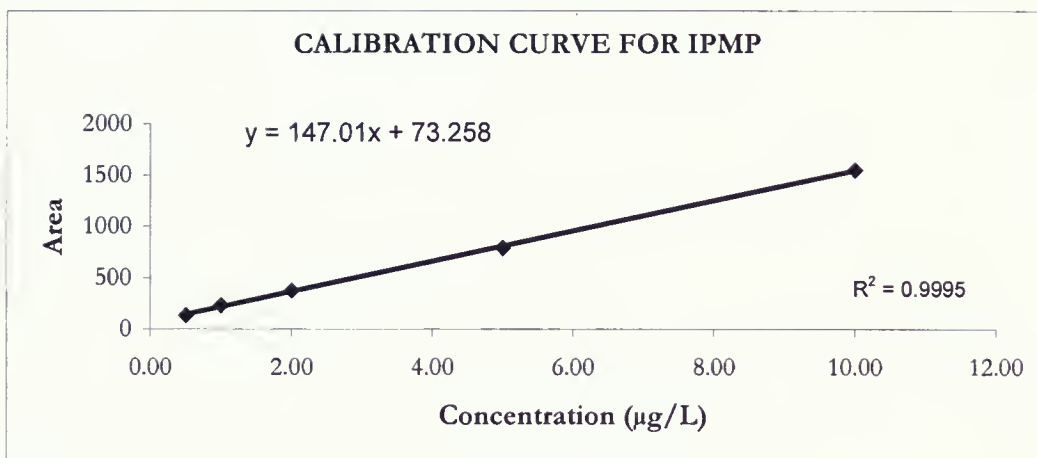
Calibration curves and results for GC/MS analyses of ladybug tainted and commercial wines samples using steam distillation method

Appendix E1 – E2:

Calibration curves and results for GC/MS analyses of Riesling barrel wine sample

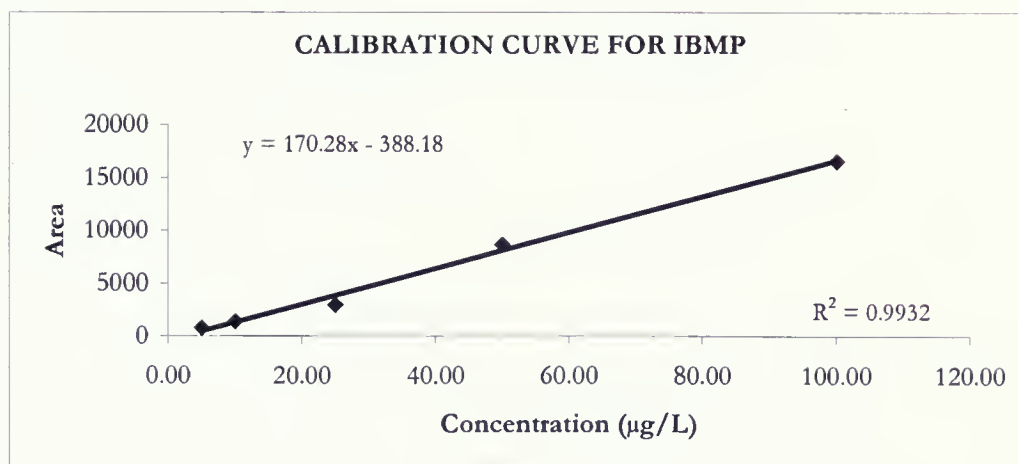
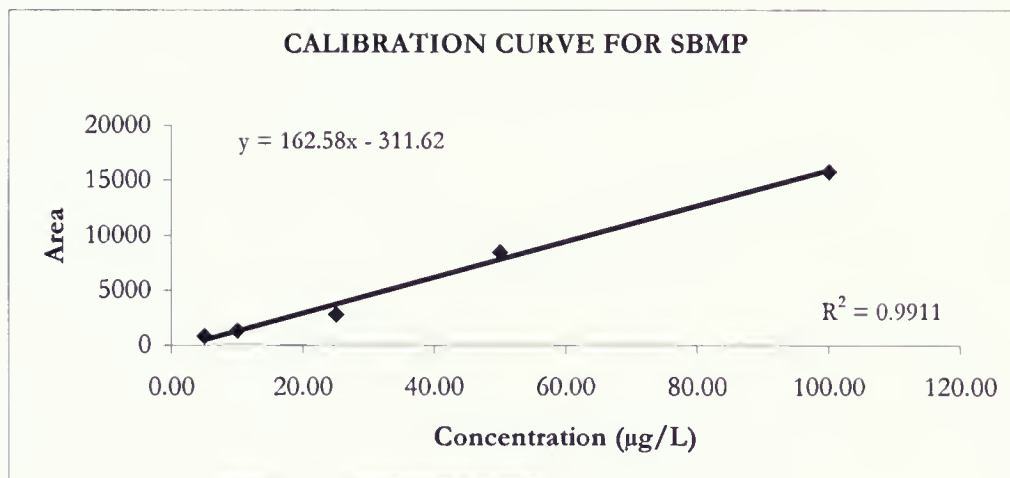
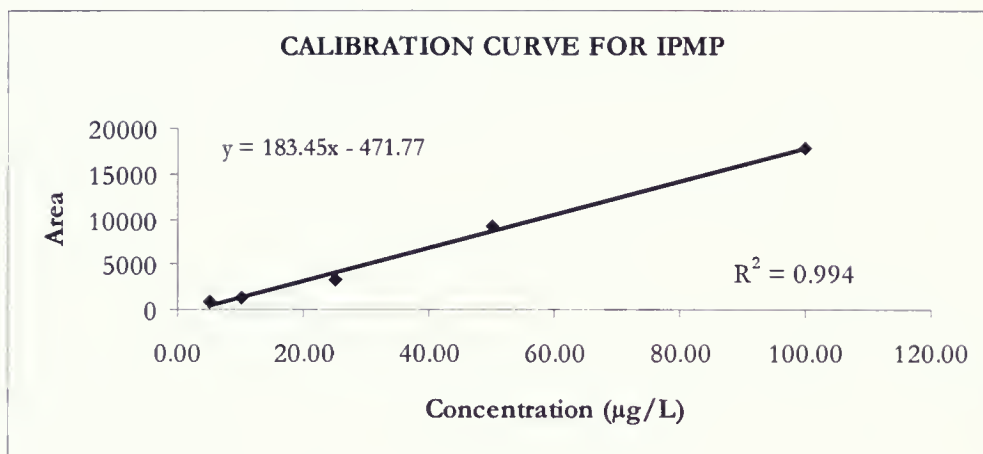
Appendix A1

Calibration Curves for HSA of ladybugs (0.5 – 10) $\mu\text{g/L}$



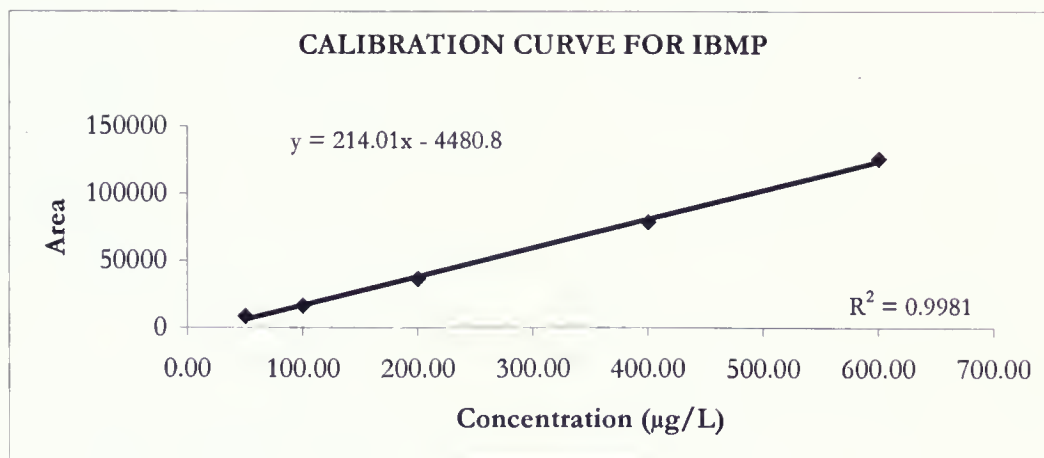
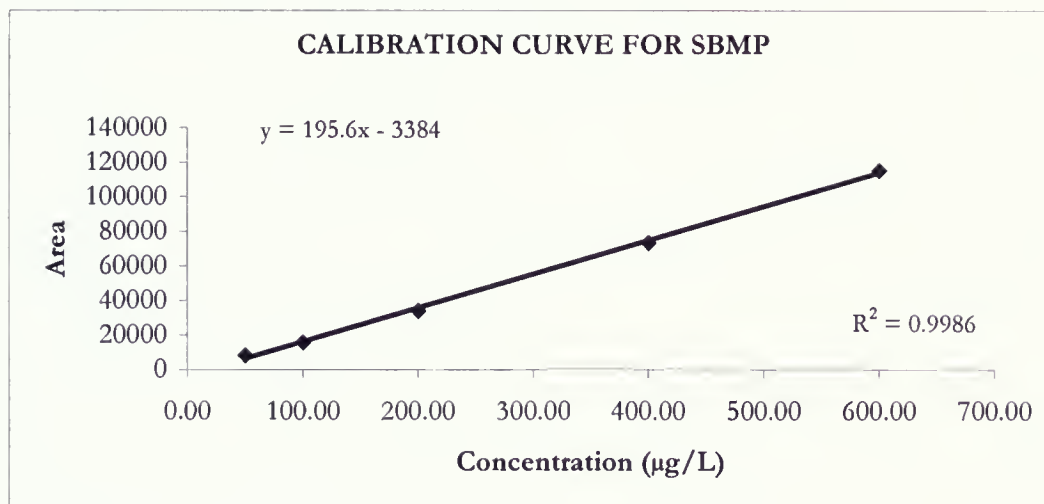
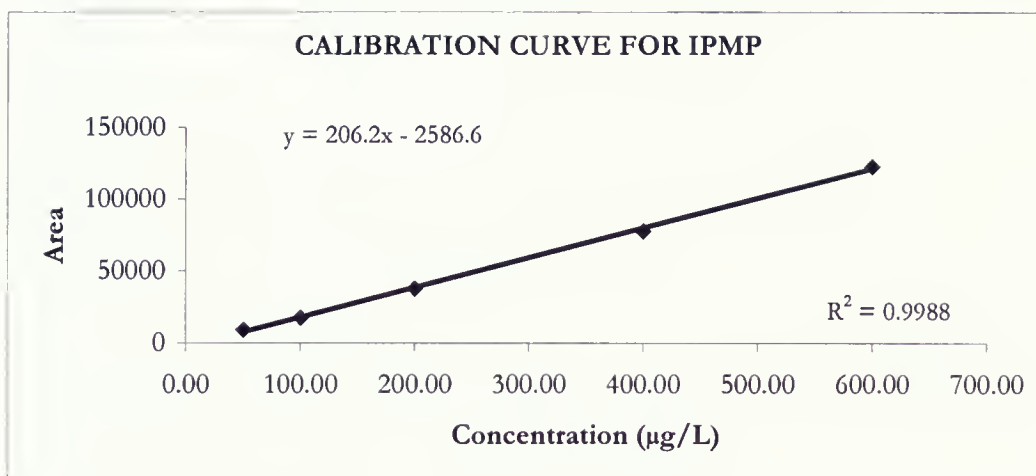
Appendix A2

Calibration Curves for HSA of ladybugs (5.0 – 100) µg/L



Appendix A3

Calibration Curves for HSA of ladybugs (50 – 600) µg/L



Appendix A4

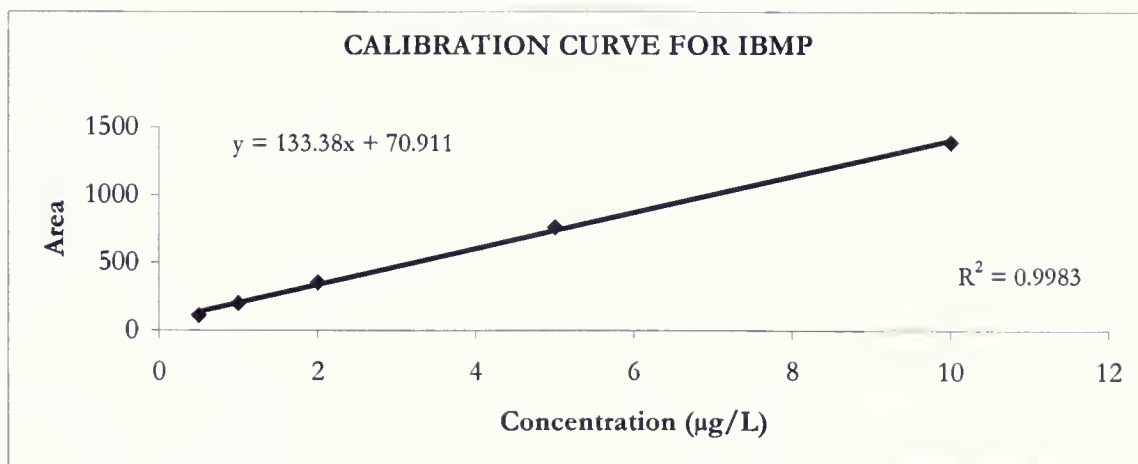
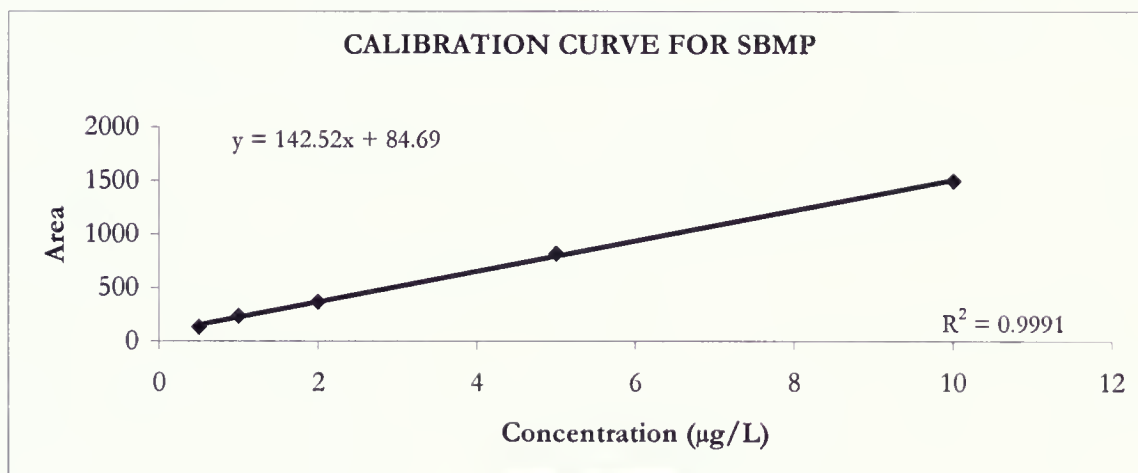
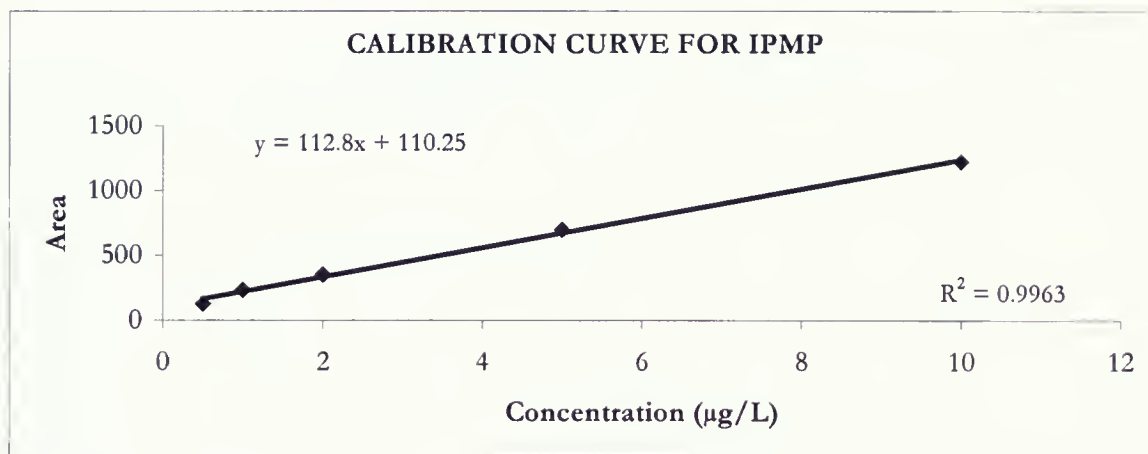
Results for headspace analyses of late summer ladybugs species

Harmonia axyridis			Hippodamia convergens			Coccinella septempunctata			
Compound	IPMP	SBMP	IBMP	IPMP	SBMP	IBMP	IPMP	SBMP	IBMP
Retention Time	10.46	11.79	11.91	10.46	11.79	11.92	10.46	11.79	11.91
Peak Area [1]	25920	1790	2400	91080	4210	14000	392	26	77
Peak Area [2]	25690	1690	2300	90880	4290	14200	388	35	78
Average Area	25810	1740	2400	90980	4250	14100	390	31	76
Standard deviation	160	70	100	140	60	140	3	6	1

Number of replicates = 2

Appendix B1

Calibration Curves for HSA of ladybugs (0.5 – 10) µg/L



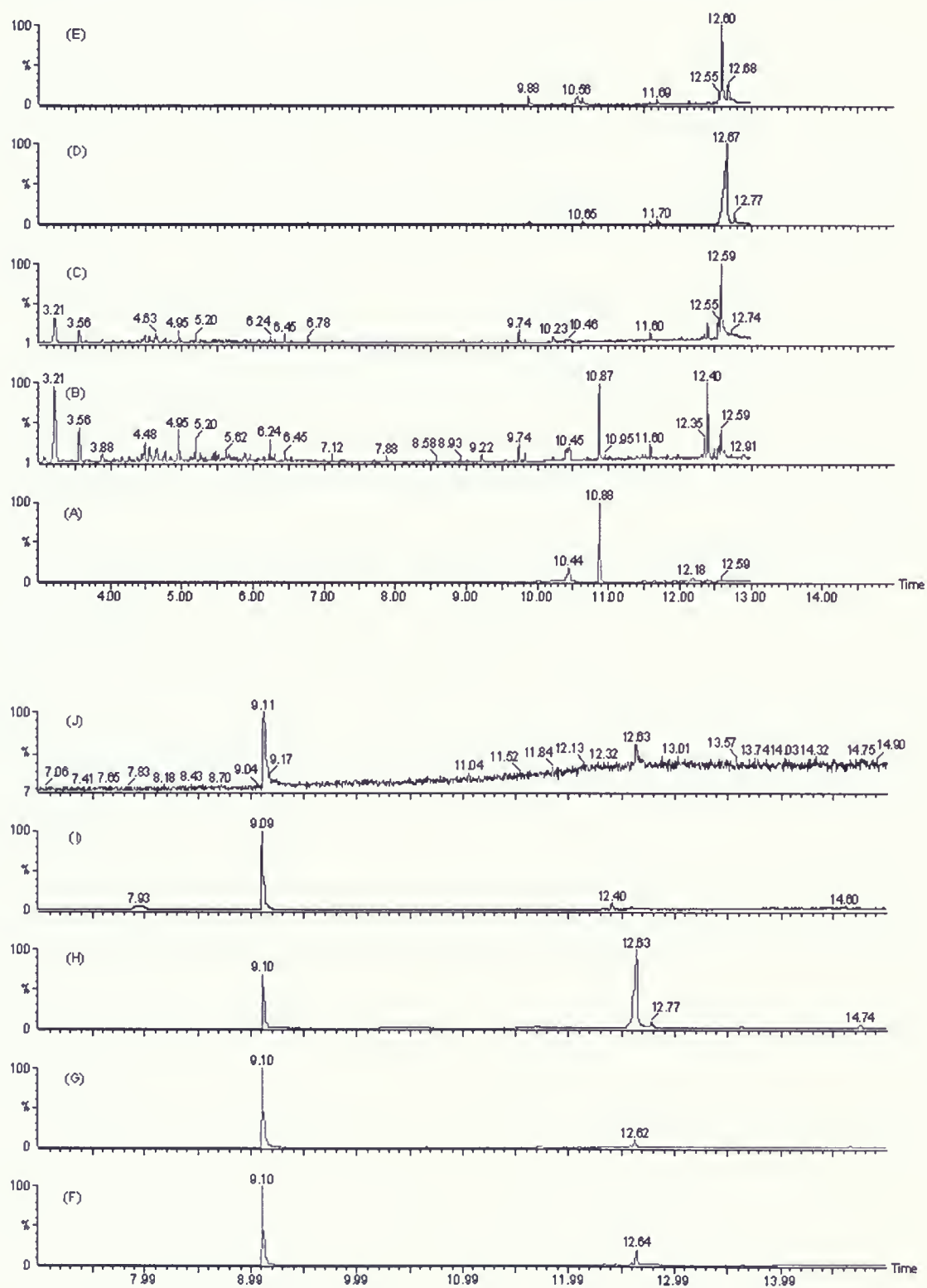
Appendix B2

Results for headspace analyses of late fall ladybugs species

Compound	<i>Harmonia axyridis</i>			<i>Coccinella septempunctata</i>		
	IPMP	SBMP	IBMP	IPMP	SBMP	IBMP
Retention Time	13.62	15.01	15.20	13.62	15.01	15.20
Peak Area [1]	743	355	312	795	216	184
Peak Area [2]	723	329	301	777	236	204
Average Area	733	340	310	790	226	194
Standard deviation	14	20	10	10	14	14

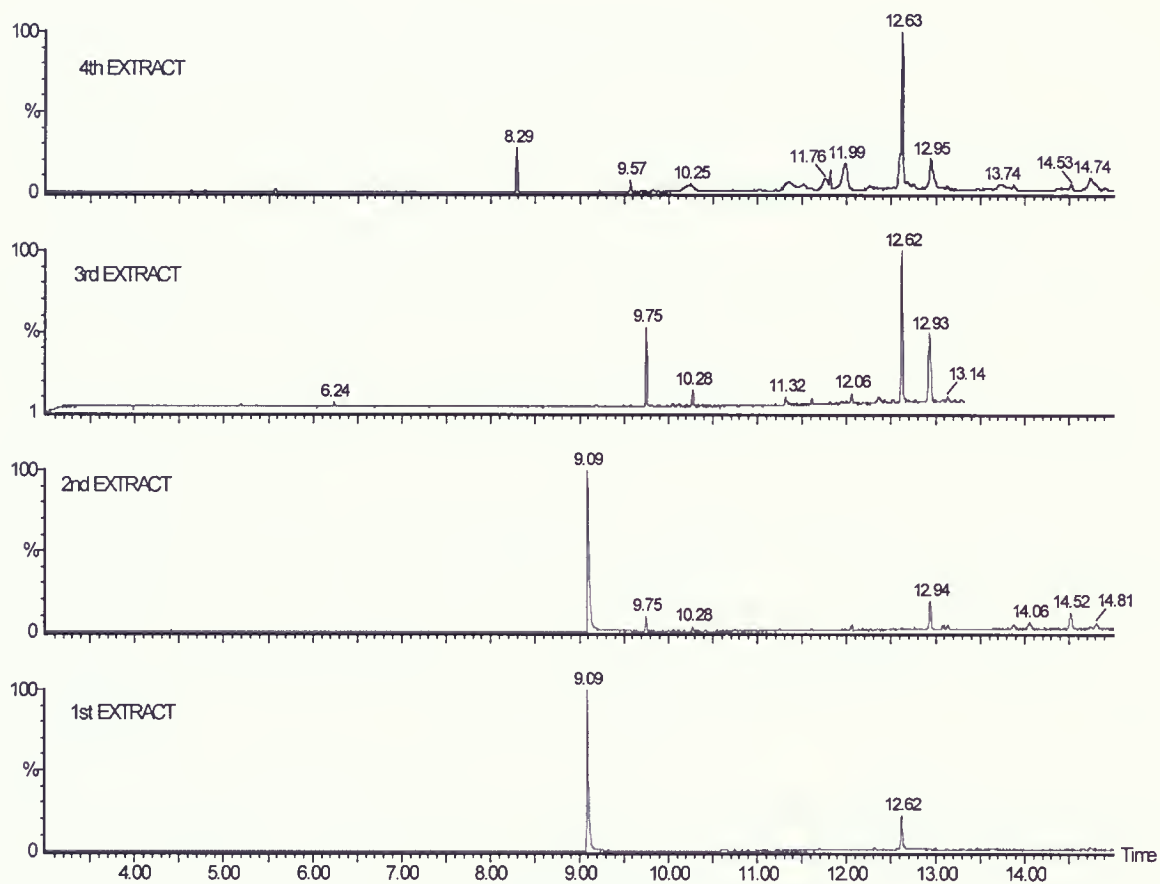
Number of replicates = 2

Appendix C1 Chromatograms for various fractions of methylene chloride extract



Appendix C2

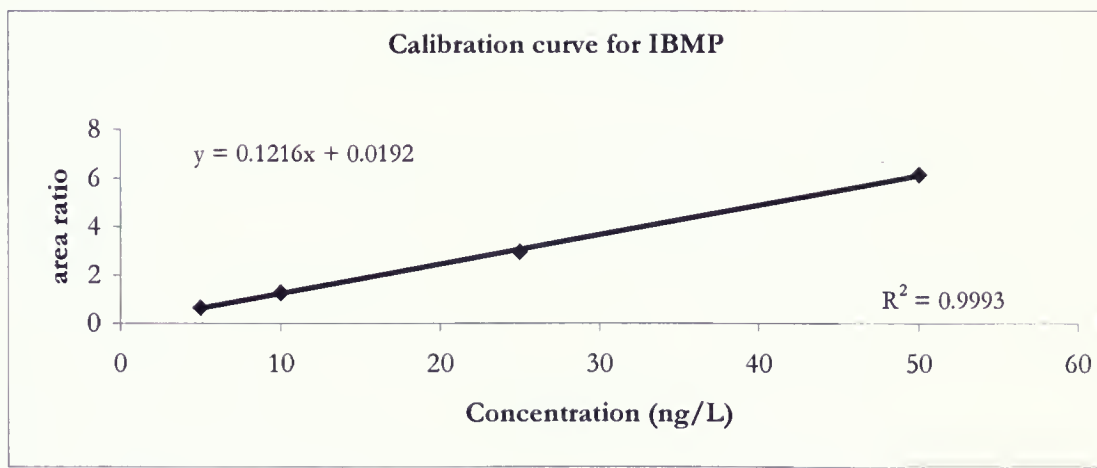
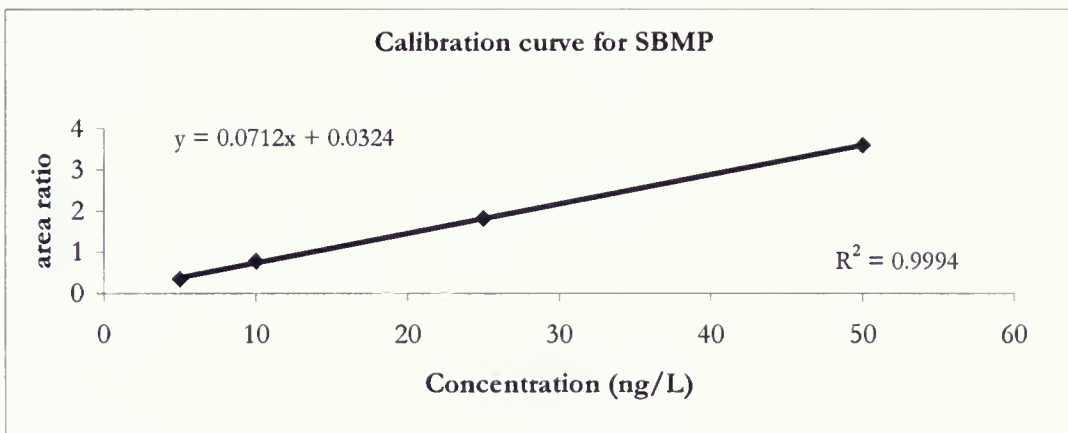
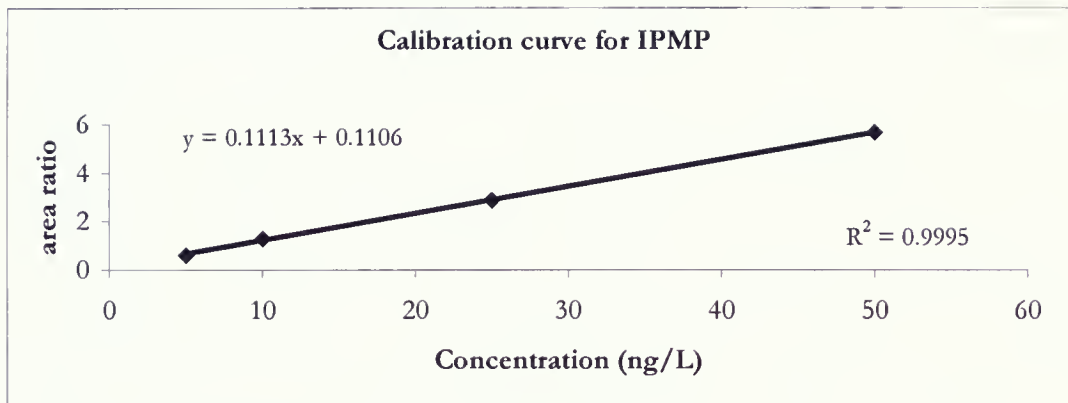
Chromatograms for extractions using Hexane and HCl



1st Extract: 0.5 M HCl; 2nd Extract: 1.0 M HCl; 3rd Extract: 2.0 M HCl; 4th Extract: Hexane

Appendix D1

Calibration Curves for Ladybug Tainted Wine Samples



Appendix D2 Results for GC/MS analyses ladybug tainted wine samples

Compound	Tainted wine sample (3 bus/L)				Tainted wine sample (10 bugs/L)				Wine Control			
	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL
Retention Time	12.41	13.71	13.83	12.37	12.41	13.63	nd	12.39	12.46	13.70	13.88	12.39
Total Peak Area	149	37	68	29	117	nd	49	31	50	29	41	29
Area Ratio	5	1	2		4	nd	2		2	1	1	
Net Area Ratio	3	0.2	0.9		3	nd	0.1		-	-	-	
Concentration (ng/L)	29.4	<5.0	7.6		8.5	nd	<5.0					

NB: Peak area: Total peak area of analyte after integration

Area Ratio: Obtained as the ratio of analyte peak area to isotope labeled peak area

Net Area Ratio: Difference between area ratio of each analyte in tainted wine and the corresponding analyte in control

DL: Deuterated labeled IPMP (internal standard)

nd: not detected

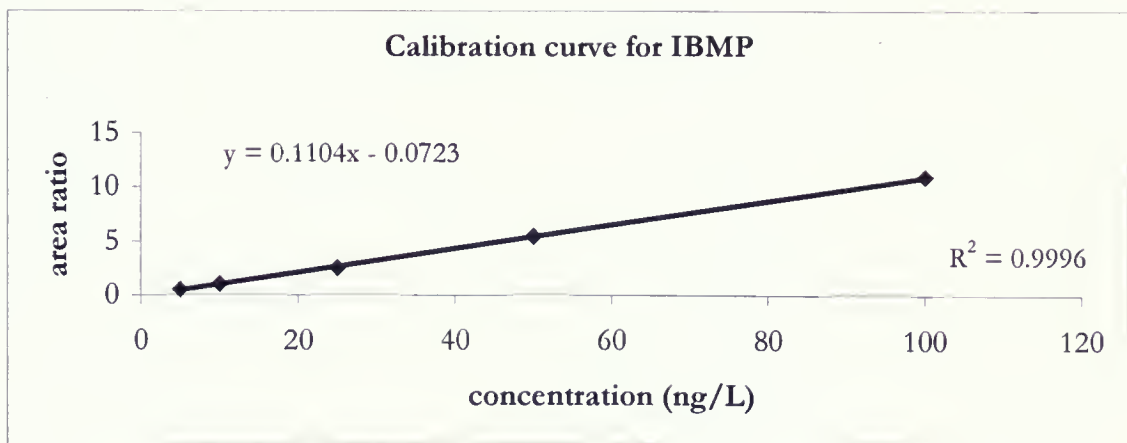
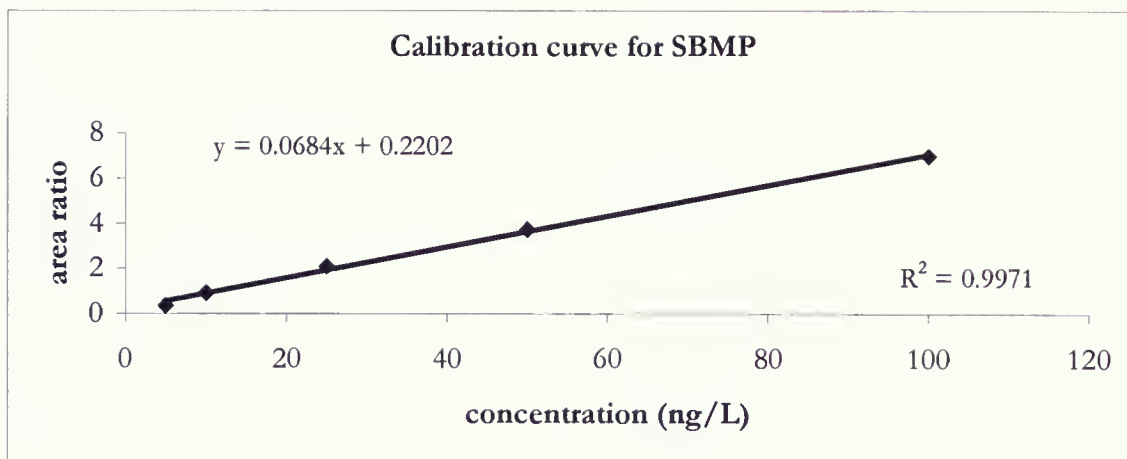
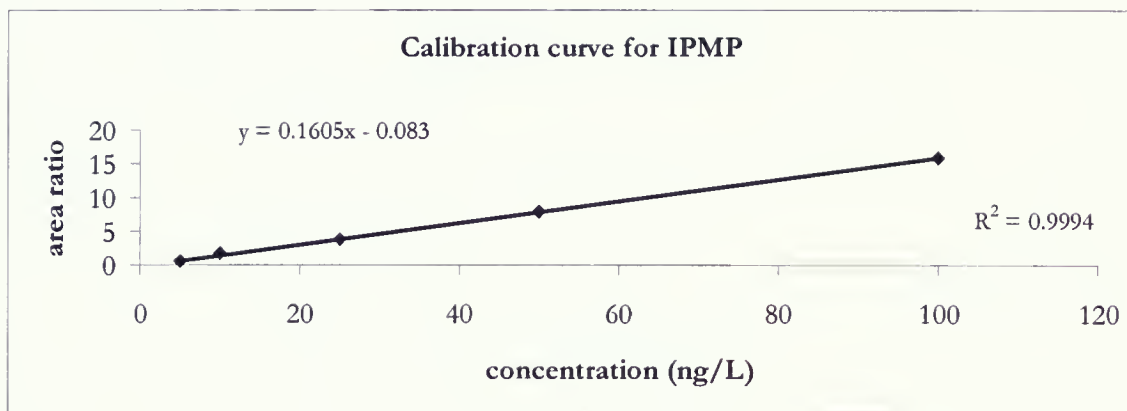
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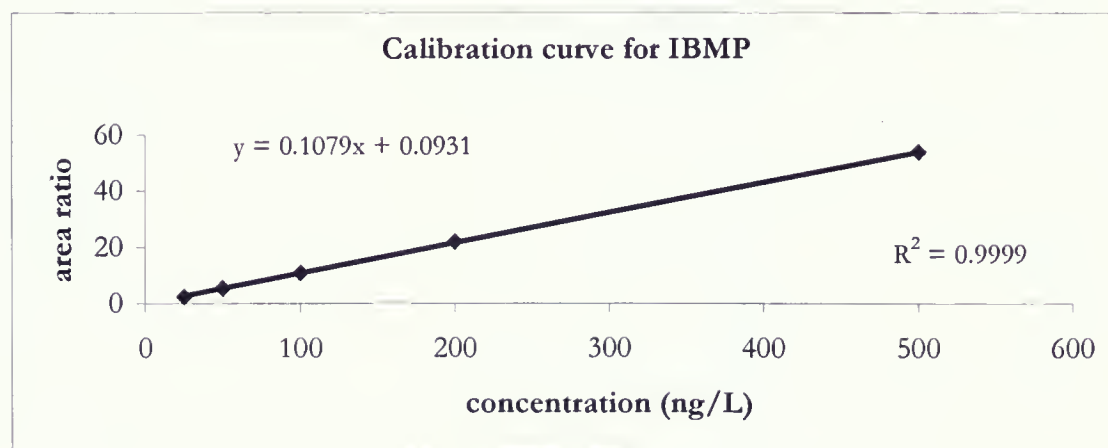
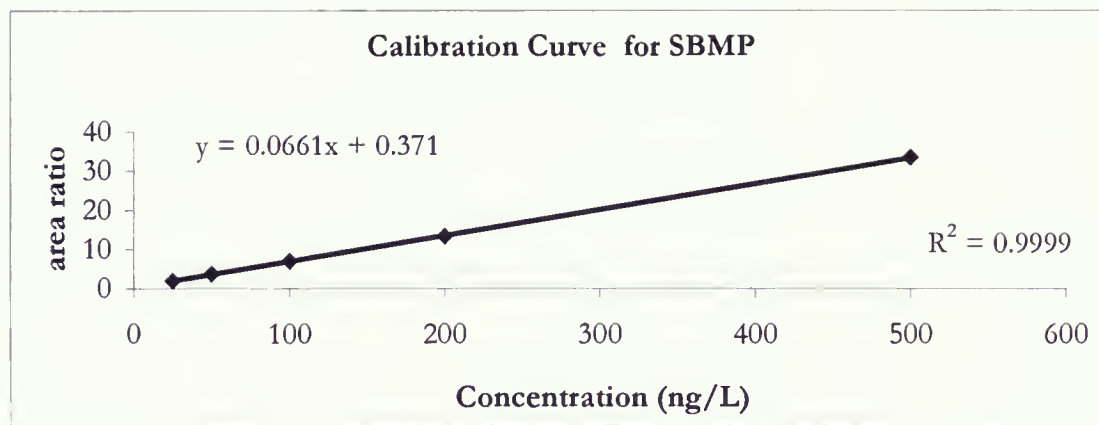
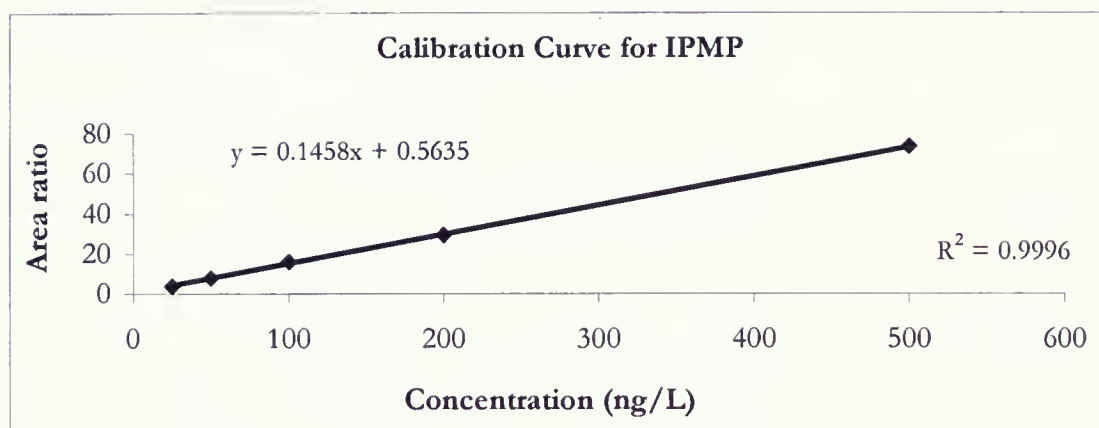
Both tainted wine samples and control were obtained from the same grape juice. As a result, area ratios obtained for the control wine sample were subtracted from that recorded for the each tainted wine sample.

Appendix D3

Calibration Curves for commercial wine samples (5 –100)

ng/L





Appendix D4 Results for GC/MS analyses of IPMP, SBMP, and IBMP in some commercial wine samples

Sample W1					Sample W2				Sample O1			
Compound	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL
Retention Time	12.46 [*]	13.72 [*]	13.88 [*]	12.39	12.46 [*]	13.70 [*]	13.88 [*]	12.40	12.46 [*]	13.70 [*]	13.88 [*]	12.39
Peak Area	412.2 [*]	224.6 [*]	145.5 [*]	29.7	498.2 [*]	111.8 [*]	306.4 [*]	27.6	714.9 [*]	132.1 [*]	234.8 [*]	30.1
Area Ratio	13.88	2.75	4.90		18.05	4.05	11.10		23.75	4.39	7.8	

Sample W3					Sample W4				Sample W5			
Compound	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL
Retention Time	12.45	13.70	13.88	12.40	12.46	13.72	13.88	12.40	12.46	13.70	13.88	12.40
	12.46	13.72	13.88	12.39	12.46	13.72	13.90	12.40	12.46	13.72	13.88	12.39
Peak Area	746	245	474	30	1114	218	381	28	780	254	746	27
	709	225	480	30	999	195	336	27	794	244	775	28
Area Ratio	25	8	16		40	8	14		29	9	28	
	24	8	16		37	7	12		28	9	28	

NB: Peak area: Total peak area of analyte after integration
Area Ratio: Obtained as the ratio of analyte peak area to isotope labeled peak area
DL: Deuterated labeled IPMP (internal standard)
nd: not detected
Values in bold print: First replicate
Values not in bold print: Second replicate
Asterix (*): Limited sample quantity (only one trial done)
Replicate number: 2

Appendix D4

Results for GC/MS analyses of IPMP, SBMP, and IBMP in some commercial wine samples

Compound	Sample W6				Sample R1				Sample R2			
	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL
Retention Time	12.46	13.78	13.88	12.41	12.48*	13.72*	13.88*	12.40	12.48*	13.72*	13.90*	12.40
	12.45	13.78	13.88	12.40								
Total Peak Area	721	nd	733	28	nd	nd	nd	29	167*	18*	66*	28
	728	nd	730	27								
Area Ratio	26	nd	26		nd	nd	nd		6	0.6	2	
	27	nd	27									

NB: Peak area: Total peak area of analyte after integration

Area Ratio: Obtained as the ratio of analyte peak area to isotope labeled peak area

DL: Deuterated labeled IPMP (internal standard)

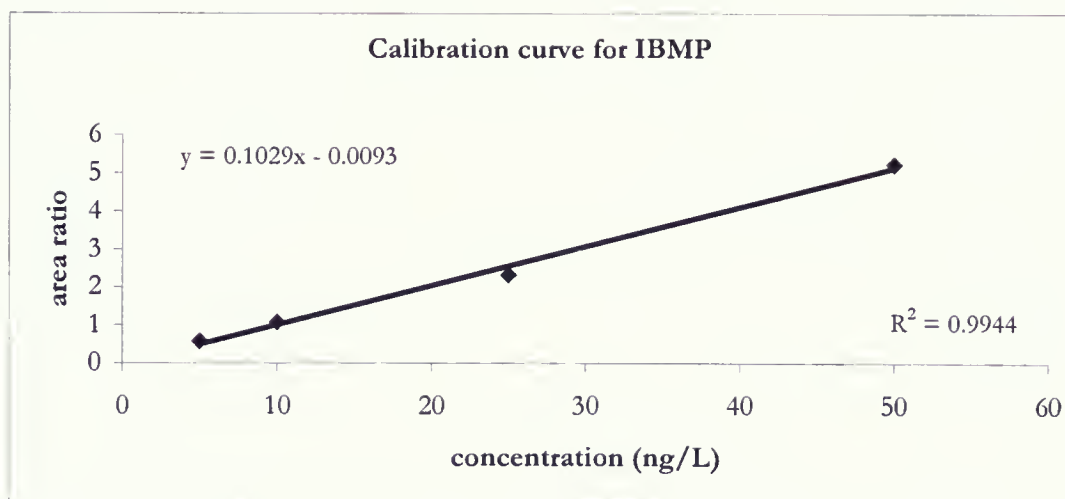
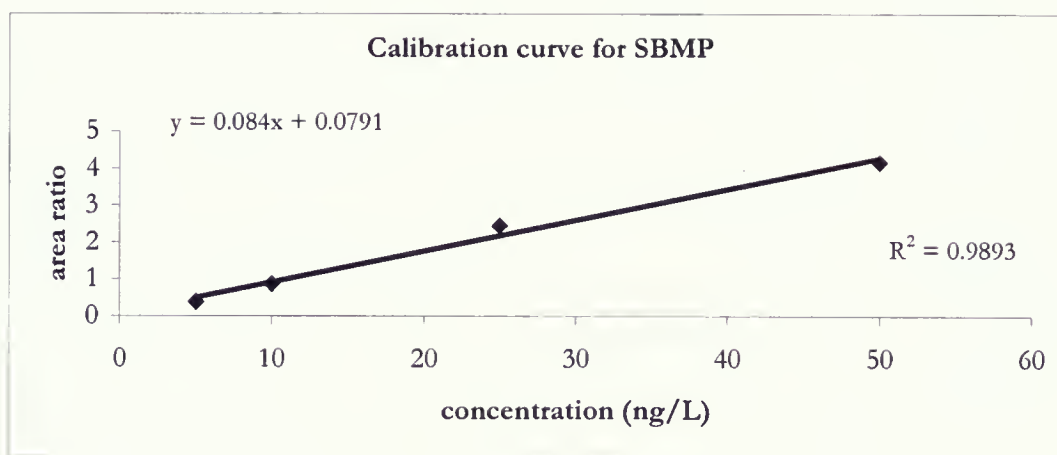
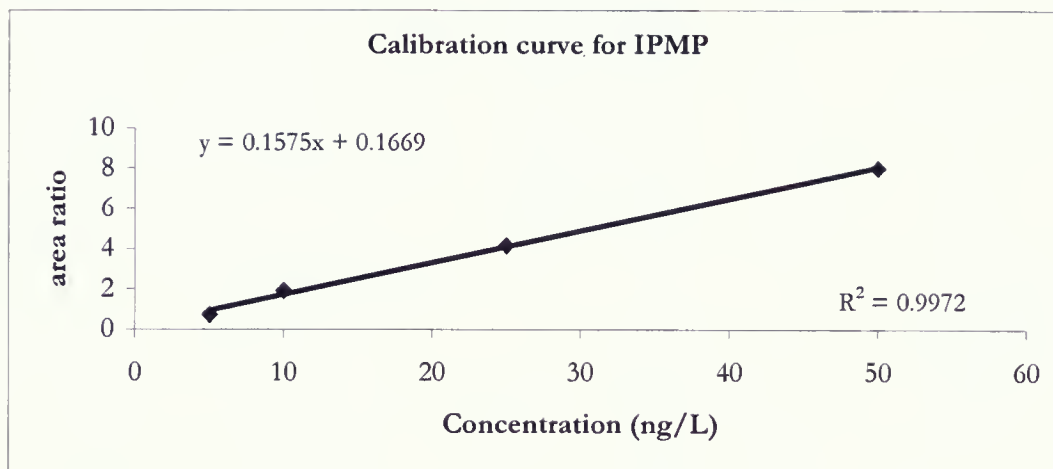
nd: not detected

Values in bold print: First replicate

Values not in bold print: Second replicate

Asterix (*): Limited sample quantity (only one trial done)

Replicate number: 2



Appendix E2

Results for GC/MS analyses of IPMP, SBMP, and IBMP in barrel wine sample

Compound	Replicate 1				Replicate 3				Replicate 3			
	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL	IPMP	SBMP	IBMP	DL
Retention Time	12.41	13.60	13.88	12.37	12.41	13.61	13.85	12.37	12.41	13.61	13.85	12.37
Peak Area	127	nd	6	28	141	nd	7	30	131	nd	7	28
Area Ratio	5	nd	0.2		5	nd	0.2		5	nd	0.3	
Concentration (ng/L)	28	nd	<5		29	nd	<5		30	nd	<5	

NB: Average concentration of IPMP: 29 ng/L.

Standard deviation: 1

nd: not detected

Replicate number: 3

