Synthesis of Isotope-labelled Methoxypyrazine Compounds as Internal Standards and Quantitative Determination of Aroma Methoxypyrazines in Water and Wines by Solid-phase Extraction with Isotope Dilution-GC-MS

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Abstract

An efficient way of synthesizing the deuterium labelled analogues of three methoxypyrazine compounds: 2-d$_3$-methoxy-3-isopropylpyrazine, 2-d$_3$-methoxy-3-isobutylpyrazine, and 2-d$_3$-methoxy-3-secbutylpyrazine, has been developed. To confirm that the deuterium labels had been incorporated into the expected positions in the molecules synthesized, the relevant characterization by NMR, HRMS and GC/MS analysis was conducted. Another part of this work involved quantitative determination of methoxypyrazines in water and wines. Solid-phase extraction (SPE) proved to be a suitable means for the sample separation and concentration prior to GC/MS analysis. Such factors as the presence of ethanol, salt, and acid have been investigated which can influence the recovery by SPE for the pyrazines from the water matrix. Significantly, in this work comparatively simple fractional distillation was attempted to replace the conventional steam distillation for pre-concentrating a sample with a relatively large volume prior to SPE. Finally, a real wine sample spiked with the relevant isotope-labelled methoxypyrazines was quantitatively analyzed, revealing that the wine with 10 beetles per litre contained 138 ppt of 2-methoxy-3-isopropylpyrazine. Interestingly, we have also found that 2-methoxy-3-secbutylpyrazine exhibits an extremely low detection limit in GC/MS analysis compared with the detection limit of the other two methoxypyrazines: 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine.
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INTRODUCTION

Methoxypyrazines in the Environment

Pyrazines have been found in a large number of foods and other natural products. Some pyrazine compounds are powerfully odorous. It is interesting that certain members of the pyrazine family are partly the sources of the pleasant aromas of roasted meats, coffee, cocoa, and cereals, while some pyrazines can be regarded as the unpleasant, musty-tasting odour-causing chemicals in water. Alkoxypyrazines are also found in raw vegetables such as peas, bell peppers, potatoes, and beets. Many organisms can produce 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine. These organisms include Actinomycete Sp., Pseudomonas taetrolens grown on milk cultures, and pseudomonas perolens grown on fish muscles. A fast increase in population of the microorganisms that produce these compounds usually brings about troubles of the odours typical of pyrazines prevailing in a certain region. For example, during the spring overflow of the North Saskatchewan River in 1990, the main source of raw water for Edmonton, large reproduction of lysobacter enzymogenes gave rise to the musty pepper odour originating from 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine since such organisms contain these pyrazines. Although a certain methoxypyrazine is a flavorant that helps establish the special aroma of some vegetables such as peas and bell peppers, its odour threshold value in water was first reported at 2 ngL\(^{-1}\) and recently recorded to reach a value as low as 0.5 ngL\(^{-1}\). The pyrazines in water could influence the water’s quality as drinking water. Therefore, effective
analytical techniques are needed to identify and quantify such low concentrations of pyrazines in water.

3-alkyl-2-methoxypyrazines have been reported at extremely low concentration (ng/L) in the list of aroma compounds of Cabernet sauvignon, Merlot noir and Sauvignon blanc wines.\textsuperscript{3-5} These pyrazines present wine tastes typical of vegetative, herbaceous and bell pepper characters.\textsuperscript{3-5} The amounts of pyrazines in wines can be very powerful in determining wine aroma. At low levels, pyrazines can be in balance with other flavour components, providing the wines a desirable flavour. However, at high levels, some pyrazines can upset the wine flavour, resulting in a bad taste of the wines. So, quantitative analysis of ultratrace pyrazines in wines becomes particularly desirable in evaluating wine qualities.

Since the amounts of methoxypyrazines in wines are of significant importance in influencing wine flavour, it becomes necessary to study where the methoxypyrazines in wines come from. Methoxypyrazines in wines originate from the grapes, whose levels of methoxypyrazines are affected by grape variety, fruit maturity, season, climate, and solar exposure of the fruit. Interestingly, a recent study\textsuperscript{15} shows that a certain kind of ladybug, \textit{Harmonia axyridis} (HA), could release its haemolymph to grapes used to make wines. Haemolymph has been verified to contain 2-isopropyl-3-methoxypyrazine.\textsuperscript{15} In this way, the methoxypyrazines could be transferred to wines by ladybugs. Such biological pathway of enriching wines with methoxypyrazines makes us think about the possible influence of HA on the sensory properties of wine.\textsuperscript{15} In this work, an analytical method was developed to determine methoxypyrazines in wines made from grape musts fermented in the presence of HA beetles.
Solid-phase Extraction

Solid-phase extraction (SPE) at present is one of the most popular techniques in sample preparation and attracts much attention from scientists working in the field of sample separation.\textsuperscript{16} Marie-Claire Hennion\textsuperscript{17} indicated that so-called disposable cartridges have been utilized for more than 20 years.\textsuperscript{17} The first cartridges were introduced in 1978.\textsuperscript{17} Syringe-format types of cartridges were developed in 1979.\textsuperscript{17} A special column directly linked to liquid chromatography (LC) was introduced in the early 1980s.\textsuperscript{17} Improvement in SPE techniques proceeded at a snail's pace for a long time and it started to get much improved during the past five or six years.\textsuperscript{17} The development of SPE resulted from the needs of decreasing volumetric usage of organic solvents.\textsuperscript{18} Also, when determining many polar analytes, it seems to be difficult to reach a good recovery by using liquid-liquid extraction (LLE).\textsuperscript{17} The reason is that many polar analytes can be partly solubilized in water and hence cannot be perfectly extracted by any organic solvent.\textsuperscript{19} SPE can overcome many disadvantages of LLE, i.e., \textit{incomplete phase separations, less-than-quantitative recoveries, use of expensive, breakable specialty glassware, and disposal of large quantities of organic solvents}.\textsuperscript{20} Operation of SPE is relatively simple with less lab time and solvent usage compared with LLE and automated SPE is feasible.\textsuperscript{20} Ability of SPE to extract polar analytes when selecting suitable sorbents also makes SPE techniques attractive to analytical chemists.\textsuperscript{17} SPE techniques are enjoying a steady increase in numbers of publications concerning the innovation on SPE sorbents or procedures.\textsuperscript{17}
Generally speaking, the mechanisms involved in SPE separation include three modes, i.e., so-called reversed phase and normal phase as well as ion exchange mode, among which the reversed phase mode was adopted in this work. In reversed phase separations, a polar (water sample) or medium polar sample which contains mid- to nonpolar analytes is passed through a nonpolar stationary phase. The alkyl- or aryl-bonded silicas are reversed phase SPE sorbent materials. Attraction between the organic analyte and the alkyl or aryl functional groups on sorbents of SPE plays a significant role in extraction of analytes from polar aqueous matrix. Such kinds of nonpolar-nonpolar attractive forces are usually regarded as van der Waals force or dispersion forces. The eluting solvent used to wash the retained analyte compounds off the sorbents of a reversed phase SPE cartridge is a kind of nonpolar solvent that can break the forces binding the compounds to the packing.

In this work, Waters Oasis silica-based SPE cartridges bonded with C_{18} sorbents were used to extract methoxypyrazines from water and wines, and to concentrate the analytes prior to GC/MS. A survey of the literature has shown that in most publications headspace solid-phase microextraction (HS-SPME) was the frequently adopted means in separation and concentration of pyrazines from a complicated sample matrix, such as wines. Only one paper described use of SPE in sample preparation in quantitative analysis of pyrazines. However, high-performance liquid chromatography (HPLC) was followed by SPE in that paper. In this work, an attempt was made to apply SPE as a means of sample preparation prior to GC/MS analysis.
Isotope Dilution Assay

So-called quantitative analysis actually means a comparative process in which the response of a purposefully added standard is compared to the response of a target species in a sample. The principles of the techniques of internal standardization involve comparing the response of two components in the same sample relative to one another. When establishing calibration curve the concentration of one component usually called reference standard is known, while the other is the target component whose concentration can be set in sample preparation. The ratio of response between these two sample components is determined with a standard run. In this manner the response factor is reported in terms of a Relative Response Factor (RRF). The calibration curve can be made according to the following expression:

\[
\frac{(\text{PeakArea})_{\text{arg std}}}{(\text{PeakArea})_{\text{std}}} = RRF \times \frac{(\text{Conc})_{\text{arg std}}}{(\text{Conc})_{\text{std}}}
\]

Generally speaking, there are three basic types of reference standards: pure analyte, chemical analog, and isotopically labelled analog. A pure analyte is the target compound itself. Chemical analogs are compounds structurally similar to the analyte, but chemically different. Isotopically labelled analogs are formed by substitution of some atoms in the analyte molecule by their "heavy" stable (non-radioactive) analogs. In many cases, carbon-12 is replaced with carbon-13, hydrogen-1 is replaced with deuterium (hydrogen-2), and nitrogen-15 is substituted for nitrogen-14.
The crucial point of using reference standard is the intentional addition of the internal standard to the sample before isolation of the analytes begins. After sample extraction and cleanup, only the ratio of response between the analyte and the internal standard needs to be measured. Then, the amount of the analyte in the sample can be determined based on the ratio of response, relative response factor, and the calibration curve. Interestingly, any losses of the analyte during the analytical procedure, such as sample preparation, injection, and instrumentation, can be taken into account in the same manner as losses of the internal standard. So it is a self-correcting system and especially applicable to trace analysis. As mentioned earlier, the concentration of 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine have been found to be as low as 2 ng L\(^{-1}\) in water.\(^{13}\) It is rather difficult to ensure no losses of the analytes at these levels during preconcentration processes.\(^1\) Under such circumstances, isotope dilution assay is recommended which actually belongs to a technique of internal standardization introducing an isotope-labelled standard directly into the analytical sample. Such technique is accurate for trace analysis but is limited by the cost and availability of labelled analogues. For quantitative analysis of methoxypyrazines at ppt levels in water and wines, a preliminary effort was made involving synthesis of three deuterated analogues of 2-methoxy-3-isopropylpyrazine (4), 2-methoxy-3-isobutylpyrazine (6), and 2-methoxy-3-secbutylpyrazine (5).

Pyrazine Synthesis
A survey of the literature has revealed that there exist several ways of constructing the pyrazine ring compounds. The earliest reports of the relevant work can be traced back to the late 1940's. These synthetic methods adopted condensation reaction to get target pyrazine ring formed. Our purpose of the envisaged project was to synthesize a library of isotope-labelled methoxypyrazine compounds into which deuterium was incorporated. To do so, it is necessary to select an appropriate synthetic approach from a limited number of synthetic schemes having been reported. In the literature,$^{25}$ a synthesis of the isotope-labelled methoxypyrazine compound (1) was described (see Figure 1).

![Figure 1. The deuterated methoxypyrazines synthesized by other workers.](image)

Unfortunately, the position at which deuteriums were incorporated was on the alkyl group rather than the methoxy group where deuterium was needed for this work. Although R. L. N. Harris, et al.$^{26}$ reported synthesis of 2-d$_3$-methoxy-3-isobutylpyrazine (2) (Figure 1), they used a tedious synthetic route involving use of a strong base NaH, and solvent THF for reaction of 2-chloro-3-isobutylpyrazine, one of crucial intermediates, with deuterated methoxide to afford the isotope-labelled alkoxy substitue on the ring (2). Our synthetic schemes were aimed to make three isotope-labelled methoxypyrazine compounds (Figure 2). The overall synthetic route was designed based mainly on the
work of H. Masuda, et al. in which they successfully synthesized non-deuterated alkoxy pyrazine compound (3) (Figure 1).

\[
\begin{align*}
\text{4} & \quad \text{5} & \quad \text{6} \\
\end{align*}
\]

Figure 2. The deuterated methoxypyrazine compounds required

Objectives

In summary, the objective of this project was the quantitative analysis of methoxypyrazines in water and wines. Since methoxypyrazines are believed to exist at ppt levels in water and wines, isotope dilution assay becomes the most accurate quantitative determination of pyrazines at such low levels. To utilize the technique of isotope dilution assay in the envisaged trace organic analysis, synthesis of a library of methoxypyrazine compounds, serving as isotope-labelled internal standards, must be the preliminary step of the research work. Upon completion of synthesis of the target isotope-labelled compounds, characterizing the deuterated compounds by such techniques as NMR, EIMS and GC/MS must be conducted to confirm the position of incorporation of the deuterium atoms in the pyrazine molecules. Worthy of emphasis is that, in this work, the corresponding methoxypyrazine compounds from Aldrich were adopted as the unlabelled references that were used to identify the synthesized labelled compounds through comparisons of NMR and EIMS data of theirs. In addition to these confirmations based
on NMR and EIMS, retention time on the GC for the labelled analogues was tested to determine if there were any significant deviations from the retention time of the Aldrich un-labelled compounds. The second part of this work involves quantitative determination of methoxypyrazines in water and wines by GC/MS coupled with isotope dilution assay which utilized the synthesized isotope-labelled methoxypyrazine compounds serving as internal standards. Prior to GC/MS analysis, isolation and preconcentration of analytes from a sample matrix were needed which involve the use of the technique of solid-phase extraction (SPE). To get a good performance of SPE, optimization of recoveries of the analytes of interest became another important task in this work. Attempts were made to investigate such effects as the presence of ethanol and salting-out on recoveries of methoxypyrazines through SPE. Our ultimate goal was to apply SPE, coupled with isotope dilution assay, to quantitatively determine the odour-causing methoxypyrazine compounds at ppt levels in water and wines.
PART ONE: Synthesis of Isotope-labelled Odour Compounds: 2-methoxy-3-isopropylpyrazine, 2-methoxy-3-isobutylpyrazine, and 2-methoxy-3-secbutylpyrazine

This part describes the synthesis of three isotope-labelled analogues of pyrazines. Characterization of the synthesized pyrazine compounds was based entirely on the corresponding NMR, MS, and GC/MS data. Moreover, the reaction mechanisms involved in the synthesis are also discussed in this part. Finally, elucidation of the fragmentation of the synthesized pyrazine compounds, under EI condition in the mass spectrometer, is given.

1.1. Experimental Methods

1.1.1. Instruments

$^1$H NMR spectra were obtained by using a Bruker Advance DP/RX 300 MHz Digital FT-NMR spectrometer. The solvent used for NMR analysis was CDCl$_3$ supplied by Isotec. Mass Spectra were obtained using a Kratos Concept 1S double. Electronic Ionization (EI) was used as the energy source for ionization in the mass spectrometer.

Gas chromatography/mass spectrometric (GC/MS) analysis was carried out using a Hewlett Packard HP gas chromatograph 5890 series fitted with a 30m × 0.25mm I.D. fused-silica column with a film thickness of 0.25 μm, coated with HP-5. The splitless injection port was heated to 275 °C. Injection (2μL) of sample in dichloromethane was done by automatic sampler. The carrier gas was helium with a pressure of 110 Kpa. The
initial column temperatures were set respectively at 70 °C for 2-methoxy-3-
secbutylpyrazine, and 50 °C for both 2-methoxy-3-isopropylpyrazine, and 2-methoxy-3-
isobutylpyrazine. The initial temperatures were held for 2 minutes. After a 2.20 minute
delay, the column temperatures were raised at rates of 10 °C min$^{-1}$ for 2-methoxy-3-
secbutylpyrazine, and 15 °C min$^{-1}$ for both 2-methoxy-3-isopropylpyrazine and 2-
methoxy-3-isobutylpyrazine. The final temperatures were 220 °C for 2-methoxy-3-
secbutylpyrazine, and 200 °C for both 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-
isobutylpyrazine. The final temperatures were held for 3 minutes. The GC was coupled to
a 5970 mass-selective detector. The interface was kept at 280 °C with the ion source
working in EI mode at 70 ev. The mass chromatograms were recorded by GC-MS
operating in the scan mode; the mass range monitored was set to 50-550 AMU.

1.1.2. Solvent preparation

The solvents that were used for the reactions were dried using the following
methods: dichloromethane was refluxed over calcium hydride; toluene was distilled.

1.1.3. Reactions

![Figure 3. Numbering of the pyrazine ring](image)

Figure 3. Numbering of the pyrazine ring
Synthesis of 3-isopropyl-2(1H)-pyrazinone (7)

The synthetic routes described by Karmas and Spoerri were adopted and modified here. A solution of valinamide (25.8 mmol, 3.0g) in methanol (60mL) in an oven dried round bottom flask was cooled to -35 °C in an absolute ethanol bath mixed with liquid nitrogen. While stirring, glyoxal (28 mmol, 3.2 mL, 40% wt. solution in water, d = 1.265 g/mL), was added rapidly. This was followed by dropwise addition of 12M NaOH solution (4.6 mL) over 1 hour while the temperature of -35 °C was still maintained. The reaction flask was maintained at this low temperature for another 1 hour. Then, it was followed by reaction at room temperature overnight under argon atmosphere. In the subsequent work-up procedures, the reaction flask was brought to 0 °C by using an ice bath and 12M HCl (4.6 mL) was added rapidly followed by addition of sodium bicarbonate (4.7g) 5 minutes later. The contents were then filtered. To the filtrate, water (60 mL) was added and then the methanol was removed by rotary evaporation. The product was extracted with dichloromethane (3 × 120 mL), then dried over anhydrous MgSO₄, and filtered. The solvent was then removed by rotary evaporation to yield 56% (2.0g, 14.5 mmol of product (7).

The product showed:

TLC: \( R_f = 0.21 \) (4% methanol/96% dichloromethane)

\(^1\)H-NMR: (CDCl₃, 300 MHz) \( \delta 1.23(6H, d, CH₃), \delta 3.36(1H, m, CH), \delta 7.10(1H, d, CH), \delta 7.36(1H, d, CH)\)

EIMS: \( m/z \) (RI%) 138([M]+, 60.4%), 123([M-Me]+, 100%), 110(40.1%), 95(51.6%)

HRMS: for C₇H₁₀N₂O, calculated 138.0794, observed 138.0789
Synthesis of 3-isobutyl-2(1H)-pyrazinone (8)

The basic procedures were the same as those for the synthesis of 3-isopropyl-2(1H)-pyrazinone (7). The starting materials were a solution of leucinamide hydrochloride (16.1 mmol, 2.1 g) and glyoxal (18 mmol, 2.1 mL), generating 1.83 g (12.0 mmol) of the product (8) with a yield of 75%.

The product showed:

TLC: \( R_f = 0.23 \) (4% methanol/96% dichloromethane)

\(^1\)H-NMR: (CDCl₃, 300 MHz) 60.75(6H, d, CH₃), 62.01(1H, m, CH), 62.45 (2H, d, CH₂), 66.93(1H, d, CH), 67.20(1H, d, CH)

HRMS: \( m/z \) (RI%) 152([M]^+, 11.2%), 137([M-Me]^+, 19.8%), 110(100%), 81(14.4%)

Synthesis of 3-secbutyl-2(1H)-pyrazinone (9)

The basic procedures were the same as those for the synthesis of 3-isopropyl-2(1H)-pyrazinone (7). The starting materials were a solution of isoleucinamide hydrochloride (16.1 mmol, 2.1 g) and glyoxal (18 mmol, 2.1 mL), generating 2.1 g (13.8 mmol) of the product (9) with a yield of 86%.

The product showed:

TLC: \( R_f = 0.22 \) (4% methanol/96% dichloromethane)

\(^1\)H-NMR: (CDCl₃, 300 MHz) 60.91(3H, t, CH₃), 61.21(3H, d, CH₃), 61.55 (1H, m, CH₂), 61.79(1H, m, CH₂), 63.29(1H, m, CH), 67.19(1H, d, CH), 67.42 (1H, d, CH)

EIMS: \( m/z \) (RI%) 152([M]^+, 14.1%), 137([M-Me]^+, 51.6%), 124(100%), 110(53.3%) 95(31.3%)

HRMS: for \( C_{8}H_{12}N_{2}O \), calculated 152.0950, observed 152.0952
Synthesis of 2-chloro-3-isopropylpyrazine (10)

The procedures described by H. Masuda, M. Yoshida and T. Shibamoto were employed and modified in this work. The compound (7) (2.0 g, 14.5 mmol) was dissolved in 5 mL of dried toluene. With stirring, phosphorus oxychloride (3.6 g, 23.6 mmol) was added rapidly. The reaction mixture was refluxed for 4.5 hours. The solvent was removed by atmospheric pressure distillation. After removal of the solvent, vacuum distillation was employed to get the target product (10) in the fraction collected. The yield of this step was 66% with 1.5 g (9.6 mmol) of the product (10). The product showed the result of TLC: $R_f = 0.74$ (4% methanol/96% dichloromethane).

Synthesis of 2-chloro-3-isobutylpyrazine (11)

The basic procedures were the same as those involved in the synthesis of 2-chloro-3-isopropylpyrazine (10). The starting materials were the compound (8) (1.8 g, 12.0 mmol) and phosphorus oxychloride (3.0 g, 19.3 mmol). The yield of this step was 54% with 1.1 g (6.5 mmol) of the product (11). The product showed the result of TLC: $R_f = 0.77$ (4% methanol/96% dichloromethane).

Synthesis of 2-chloro-3-secbutylpyrazine (12)

The basic procedures were the same as those involved in the synthesis of 2-chloro-3-isopropylpyrazine (10). The starting materials were the compound (9) (2.1 g, 13.8 mmol) and phosphorus oxychloride (3.4 g, 22.5 mmol). The yield of this step was 51% with 1.2
g (7.0 mmol) of the product (12). The product showed the result of TLC: \( R_f = 0.76 \) (4% methanol/96% dichloromethane).

**Synthesis of 2-d3-methoxy-3-isopropylpyrazine (4)**

A pea-sized piece of sodium was immersed in 10 g of methyl alcohol-d4 under argon atmosphere. Sodium methylate-d3 (NaOCD3) in methyl alcohol-d4 solution was generated by the exothermal reaction in a cold water bath. The resultant solution was rapidly added to the chloro derivative (10) and the reaction mixture was refluxed for 5~6 hours. Then, the resulting mixture was added with 10 mL of distilled water. The product was extracted with ethyl acetate (3 × 10 mL), then dried over anhydrous MgSO4, and filtered. The product was purified by rotary evaporation to obtain amount of 1.3 g (8.4 mmol) of the product (4) in 87% yield (relative to the quantity of 10).

The product showed:

**TLC:** \( R_f = 0.89 \) (4% methanol/96% dichloromethane)

**\( ^1 \)H-NMR:** (CDCl3, 300 MHz) \( \delta 1.26 \) (6H, d, CH3), \( \delta 3.36 \) (1H, m, CH), \( \delta 7.91 \) (1H, d, CH), \( \delta 8.04 \) (1H, d, CH)

**EIMS:** m/z (RI%) 155([M]+, 41.4%), 140([M-Me]+, 100%), 127(26.6%), 105(17.4%), 95(17.3%)

**HRMS:** for C8H9D3N2O, calculated 155.1250 \(^{29}\), observed 155.1141

**Synthesis of 2-d3-methoxy-3-isobutylypyrazine (6)**

The basic procedures were the same as those involved in the synthesis of 2-d3-methoxy-3-isopropylpyrazine (4). The starting materials were the chloro derivative (11)
and the sodium methylate-d₃ (NaOCD₃) generated prior to this step, affording an amount of 0.8 g (4.7 mmol) of the product (6) in 72% yield (relative to the quantity of 11).

The product showed:

TLC: \( R_f = 0.92 \) (4% methanol/96% dichloromethane)

\(^{1}\)H-NMR: (CDCl₃, 300 MHz) 80.94 (6H, d, CH₃), 82.17 (1H, m, CH), 82.69 (2H, d, CH₂), 87.92 (1H, d, CH), 88.03 (1H, d, CH)

EIMS: m/z (RI%) 169([M]^+, 4.2%), 154([M-Me]^+, 17.7%), 127(100%), 109(3.0%), 95(15.3%)

HRMS: for C₉H₁₄D₃N₂O, calculated 169.1407, observed 169.1293

**Synthesis of 2-d₃-methoxy-3-secbutylpyrazine (5)**

The basic procedures were the same as those involved in the synthesis of 2-d₃-methoxy-3-isopropylpyrazine (4). The staring materials were the chloro derivative (12) and the sodium methylate-d₃ (NaOCD₃) generated prior to this step, affording an amount of 0.9 g (5.3 mmol) of the product (5) in 76% yield (relative to the quantity of 12).

The product showed:

TLC: \( R_f = 0.90 \) (4% methanol/96% dichloromethane)

\(^{1}\)H-NMR: (CDCl₃, 300 MHz) 80.86 (3H, t, CH₃), 81.23 (3H, d, CH₃), 81.60 (1H, m, CH₂), 81.80 (1H, m, CH₂), 83.15 (1H, m, CH), 87.91 (1H, d, CH), 88.06 (1H, m, CH)

EIMS: m/z (RI%) 169([M]^+, 4.7%), 154([M-Me]^+, 42.7%), 141(100%), 127(55.2%), 105(12.6%), 95(7.6%)

HRMS: for C₉H₁₁D₃N₂O, calculated 169.1407, observed 169.1292
1.2. Results and Discussion

1.2.1. Reaction mechanisms

The overall synthesis of isotope-labelled methoxypyrazines includes three major steps. The first step involved a condensation of a dicarbonyl with a d-amino acid amide, giving rise to formation of 2-pyrazinone intermediates. The second step was dedicated to formation of 2-chloropyrazine derivatives through reactions of the 2-pyrazinones (the step 1 intermediates) with phosphorus oxychloride. In the subsequent step, the 2-chloropyrazine derivatives (the step 2 intermediates) were reacted with deuterated sodium methoxide to afford the desired isotope-labelled methoxypyrazines. The mechanism of the first step used to get methoxypyrazinone (the ring system) formed can be found in D. A. Gerritsma’s thesis.\textsuperscript{30} In this work, steps 2 and 3 will be discussed in terms of mechanistic elucidation.

Prior to mechanistic studies of the latter two steps, it is particularly necessary to emphasize that the step 1 product could not be a single compound. The Karmas-Spoerri reaction proved to form two isomers, which coexist in equilibrium shown below:

\[ \text{13} \quad \text{14} \]
It is argued that the 2-pyrazinone (13) is energetically more favourable than the corresponding 2-hydroxypyrazine (14). This assumption is based on the fact that immediate methylation of the step 1 intermediate has been proved to be impossible in making deuterium incorporated into the desired position on the pyrazine molecules. In other words, transformation of the 2-pyrazinone into the 2-hydroxypyrazine seems to be most desirable in synthesis of methoxypyrazines. To achieve this conversion, formation of a particular chloro-substituted pyrazine becomes crucial in the total synthesis scheme. With the chloro derivatives in hand, deuterium incorporation into the methoxy group can be realized by chloride displacement by the deuterated methoxide. The overall mechanism is shown in Figure 4, which describes the formation of the chloro pyrazine and the methylation of the chloro derivative.
Figure 4. Reaction mechanism for the formation of the chloro pyrazine and the synthesis of the deuterated pyrazine

As the oxygen atom in the hydroxide group in molecule (14) has stronger nucleophilicity than that in the carbonyl group in molecule (13), phosphorus oxychloride compound can more readily react with compound (14) than with compound (13). The strong interaction between the molecule (14) and phosphorus oxychloride drives the reaction to completion, resulting in formation of the chloro pyrazine which is then transferred to the desired isotope-labelled methoxypyrazine via replacement of the halogen with an alkoxide group containing deuterium.

1.2.2. Characterization of the synthesized isotope-labelled pyrazine compounds

To confirm the success of the synthesis of the isotope-labelled methoxypyrazine compounds requires comparison of the synthesized analogues with the corresponding non-deuterated pyrazine compounds available from Aldrich in terms of NMR, MS and GC/MS analysis. Only with these comparative data obtained on a basis of taking the Aldrich non-deuterated pyrazines as references can one absolutely ensure the availability of the synthesized deuterated methoxypyrazines. Tables 1, 2 and 3 give the $^1$H-NMR data of the Aldrich non-deuterated pyrazine compounds and the isotope-labelled analogues. A simple look at these tables indicates that the $^1$H-NMR chemical shifts of the
Table 1.

<table>
<thead>
<tr>
<th>Aldrich</th>
<th>δ1.26(6H,d,CH₃)</th>
<th>δ3.36(1H,m,CH)</th>
<th>δ3.96(3H,s,CH₃)</th>
<th>δ7.91(1H,d,CH)</th>
<th>δ8.04(1H,d,CH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Made</td>
<td>δ1.26(6H,d,CH₃)</td>
<td>δ3.36(1H,m,CH)</td>
<td>absent (3D, OCD₃)</td>
<td>δ7.91(1H,d,CH)</td>
<td>δ8.04(1H,d,CH)</td>
</tr>
</tbody>
</table>

Table 2.

<table>
<thead>
<tr>
<th>Aldrich</th>
<th>δ0.94(6H, d, CH₃)</th>
<th>δ2.17(1H, m, CH)</th>
<th>δ2.69(2H, d, CH₂)</th>
<th>δ3.96(3H, s, CH₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Made</td>
<td>δ0.94(6H, d, CH₃)</td>
<td>δ2.17(1H, m, CH)</td>
<td>δ2.69(2H, d, CH₂)</td>
<td>absent (3D, OCD₃)</td>
</tr>
<tr>
<td>Aldrich</td>
<td>δ7.92(1H, d, CH)</td>
<td>δ8.03(1H, d, CH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Made</td>
<td>δ7.92(1H, d, CH)</td>
<td>δ8.03(1H, d, CH)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.
<table>
<thead>
<tr>
<th>Aldrich</th>
<th>δ0.86(3H, t, CH₃)</th>
<th>δ1.23(3H, d, CH₃)</th>
<th>δ1.60(1H, m, CH₂)</th>
<th>δ1.80(1H, m, CH₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Made</td>
<td>δ0.86(3H, t, CH₃)</td>
<td>δ1.23(3H, d, CH₃)</td>
<td>δ1.60(1H, m, CH₂)</td>
<td>δ1.80(1H, m, CH₂)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aldrich</th>
<th>δ3.15(1H, m, CH)</th>
<th>δ3.96(3H, s, CH₃)</th>
<th>δ7.91(1H, d, CH)</th>
<th>δ8.06(1H, m, CH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Made</td>
<td>δ3.15(1H, m, CH)</td>
<td><strong>absent</strong> (3D, OCD₃)</td>
<td>δ7.91(1H, d, CH)</td>
<td>δ8.06(1H, m, CH)</td>
</tr>
</tbody>
</table>

Hydrogen in the synthesized compounds and the corresponding Aldrich compounds have almost identical values and patterns. The huge difference between the two groups of pyrazine compounds is just the disappearance of hydrogen peaks of the methoxy group in the deuterated analogues because of substitution of deuterium for hydrogen. This provides strong evidence of deuterium incorporation into the methoxy group.

Table 4. Comparison between calculated FM and observed FM by HRMS

<table>
<thead>
<tr>
<th></th>
<th>non-deuterated isopropyl pyz (Aldrich)</th>
<th>deuterated isopropyl pyz (synthesized)</th>
<th>non-deuterated isobutyl pyz (Aldrich)</th>
<th>deuterated isobutyl pyz (synthesized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated FM²⁹,³¹</td>
<td>152.0950</td>
<td>155.1250</td>
<td>166.1107</td>
<td>169.1407</td>
</tr>
<tr>
<td>Observed FM</td>
<td>152.0953</td>
<td>155.1141</td>
<td>166.1108</td>
<td>169.1293</td>
</tr>
<tr>
<td>Relative deviation (%)</td>
<td>0.0002</td>
<td>0.007</td>
<td>0.0001</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>non-deuterated secbutyl pyz (Aldrich)</td>
<td>deuterated secbutyl pyz (synthesized)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------------------------------------</td>
<td>---------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calculated FM&lt;sup&gt;29,31&lt;/sup&gt;</td>
<td>166.1107</td>
<td>169.1407</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed FM</td>
<td>166.1103</td>
<td>169.1292</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative Deviation (%)</td>
<td>0.0002</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4 shows the HRMS data of the Aldrich non-deuterated pyrazine compounds and the isotope-labelled analogues. By comparison, it is clear that the mass spectra of these two groups of pyrazine compounds exhibit the similar patterns, they just vary by 3 AMU for each corresponding peak. This also helps confirm the synthesized isotope-labelled pyrazines. Finally, GC/MS analysis was performed to determine whether or not

Table 5. Retention times of methoxypyrazine compounds (Aldrich vs. the synthesized vs. the mixture of the both) in GC/MS

<table>
<thead>
<tr>
<th></th>
<th>Isopropyl pyrazine</th>
<th>Isobutyl pyrazine</th>
<th>Secbutyl pyrazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrich (reference)</td>
<td>6.90 min</td>
<td>7.91 min</td>
<td>7.38 min</td>
</tr>
<tr>
<td>Synthesized (labelled)</td>
<td>6.88 min</td>
<td>7.89 min</td>
<td>7.36 min</td>
</tr>
</tbody>
</table>
the isotope-labelled compounds could serve as analyte analogue in isotope dilution assay. 

Table 5 lists the retention times of the three pairs of pyrazine compounds. In a pair of the compounds, one is the synthesized labelled compound and another is the corresponding non-deuterated Aldrich compound. Obviously, these data show that the GC retention times of the synthesized and of the Aldrich compounds do agree within about ±1.2 sec, which confirms the acceptable use of the synthesized isotope-labelled analogues for isotope dilution assay.

### 1.2.3. Fragmentation patterns in mass spectrometry

The synthesis of labelled compounds provides one not only the source of internal standards used in isotope dilution assay but also a clue with which we could elucidate how the molecule of interest fragments in the mass spectrometer (refer to Table 6). This is especially significant if needed to determine a reasonable way of fragmentation when

Table 6. Fragment peaks characteristic of the deuterated compounds and worthy of interpretation of their formation in the mass spectrometer.

<table>
<thead>
<tr>
<th>Isobutylpyrazine</th>
<th>m/z</th>
<th>154</th>
<th>127</th>
<th>109</th>
<th>95</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI%*</td>
<td></td>
<td>17.7</td>
<td>100.0</td>
<td>3.0</td>
<td>15.3</td>
</tr>
<tr>
<td>secbutylpyrazine</td>
<td>m/z</td>
<td>105</td>
<td>154</td>
<td>141</td>
<td>127</td>
</tr>
<tr>
<td>RI%*</td>
<td></td>
<td>12.6</td>
<td>42.7</td>
<td>100.0</td>
<td>55.2</td>
</tr>
<tr>
<td>isopropylpyrazine</td>
<td>m/z</td>
<td>105</td>
<td>140</td>
<td>141</td>
<td>127</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>RI%*</td>
<td></td>
<td>17.4%</td>
<td>100.0%</td>
<td>15.3%</td>
<td>26.6%</td>
</tr>
</tbody>
</table>

* relative intensity of a peak of interest in percentage of the base peak.

there maybe exist different pathways of fragmentation but giving the same m/z value (see Figure 5). The first challenging puzzle is related to the first major fragmentation that involves the loss of a methyl group. For example, a methyl group loss from non-deuterated 2-methoxy-3-isopropylpyrazine leads to a base peak at m/z = 137; while a methyl group loss from either non-deuterated 2-methoxy-3-isobutylpyrazine or non-deuterated 2-methoxy-3-secbutylpyrazine gives rise to a peak at m/z = 151. Interestingly, there are two likely pathways for each compound of the three pyrazines to be responsible for this loss (see Figure 5). An examination of those molecular structures reveals that such first methyl loss may come from either the methoxy methyl group or the alkyl side chain. It is obvious that the synthesized deuterated pyrazine compounds can be employed to figure out which pathway should be reasonable for such methyl loss since the incorporation of the deuterium in the methoxy methyl group provides a clear distinction between the methoxy methyl group and the alkyl side methyl group. If the first mode of fragmentation were adopted, the loss of the methyl group (-CD3) would account for a large peak at m/z = 151 for both 2-d3-methoxy-3-isobutylpyrazine and 2-d3-methoxy-3-secbutylpyrazine,
Figure 5. CH₃ loss from the three non-deuterated methoxypyrazine molecules.
and for a base peak at m/z = 137 for 2-d3-methoxy-3-isopropylpyrazine. However, their mass spectra clearly indicate that no peaks were found relevant to the cations resulted from the loss of the methoxy methyl group. Consequently, only the loss of the methyl group from the alkyl side chain can lead to a large peak at m/z = 154 for both 2-d3-methoxy-3-isobutylpyrazine and 2-d3-methoxy-3-secbutylpyrazine (Figure 6). For 2-d3-methoxy-3-isopropylpyrazine, it is the loss of the methyl group from the alkyl side chain to give rise to the base peak at m/z = 140 (Figure 6). Interestingly, our mass spectra have completely got rid of the possibility of the loss of the methyl group from the deuterium label incorporated in the methoxy methyl group. This result is remarkably different from that obtained in D. A. Gerritsma’s thesis in which the loss of the methyl group was supposed to be either from deuterium label or from the alkyl side chain.

An another profoundly important fact, we observed, is that the three isotope analogues have similar fragmentation patterns in mass spectrometry to those found in the corresponding Aldrich compounds, but just differ by 3 AMU between each pair. The difference of 3 AMU is attributed to the substitution of deuterium for hydrogen. So, any
interpretation of fragmentation for the isotope-labelled analogues can also

\[
\begin{align*}
\text{m/z = 169} & & \text{m/z = 154} \\
\text{m/z = 169} & & \text{m/z = 154} \\
\text{m/z = 155} & & \text{m/z = 140}
\end{align*}
\]

Figure 6. CH$_3$ loss from the three deuterated methoxypyrazine molecules.

apply to the non-deuterated Aldrich compounds. Consequently, it is such similarities in fragmentation patterns between the labelled analogues and the non-deuterated Aldrich standards to offer strong support in confirmation of the isotope-labelled analogues synthesized in this work.
Figure 7. Formation of masses 127, 109 and 95 from deuterated 2-methoxy-3-isobutylpyrazine.

Fragmentation involving a McLafferty rearrangement can be used to account for the base peaks and other significant peaks in mass spectra of both 2-d$_3$-methoxy-3-isobutylpyrazine and 2-d$_3$-methoxy-3-secbutylpyrazine. Figure 7 indicates that the base peak at m/z = 127 in the mass spectrum of 2-d$_3$-methoxy-3-isobutylpyrazine is formed by a McLafferty rearrangement with a loss of propene C$_3$H$_6$. A further cleavage of the base fragment leads to either a peak at m/z = 109 or another peak at m/z = 95, the latter involving a migration of a deuterium from the methoxy methyl group to the ring and a formaldehyde loss (Figure 7).
Similarly, the base peak at $m/z = 141$ for 2-d$_3$-methoxy-3-secbutylpyrazine can be attributed to a McLafferty rearrangement with a loss of ethene C$_2$H$_4$ (Figure 8). The other major peak at $m/z = 127$ is due to the loss of methylene$^{30}$ and a hydrogen shift (Figure 8). A deuterium shift and subsequent formaldehyde loss would afford a compound with a peak at $m/z = 95$ (Figure 8). Gerritsma proposed an explanation for the large peak at $m/z = 127$ in the mass spectrum of 2-d$_3$-methoxy-3-isopropylpyrazine. $^{30}$ It involves two methylene losses and a hydrogen shift as shown in Figure 9, which afford the large peak at $m/z = 127$ and another peak at $m/z = 141$. Further fragmentation includes a migration of deuterium from the methoxy methyl group to the ring and subsequent formaldehyde loss, giving rise to a peak at $m/z = 95$ (Figure 9). The peak at $m/z = 95$ could be regarded
as a characteristic peak representing the pyrazine ring with a deuterium label since this peak is observed in all the three isotope-labelled compounds.

![Diagram of molecular structures](image)

Figure 9. Formation of masses 141, 127 and 95 from deuterated 2-methoxy-3-isopropylpyrazine.

An interesting difference among the mass spectra of the three isotope analogues is that a peak at m/z = 105 can be found with both 2-d3-methoxy-3-isopropylpyrazine and 2-d3-methoxy-3-secbutylpyrazine but, no such a peak with 2-d3-methoxy-3-isobutylpyrazine. As shown in Figure 10, a significant fragmentation could occur involving a hydrogen shift and a loss of methane or ethane as well as a loss of radical – OCD3, which would lead to the peak at m/z = 105. However, a simple glance at
molecular structure of 2-d₃-methoxy-3-isobutylpyrazine reveals that there is no methyl group at the alpha carbon and hence no corresponding fragmentation similar to the above-mentioned can occur. As a result, the peak at m/z = 105 cannot be observed in mass spectrum of 2-d₃-methoxy-3-isobutylpyrazine.
PART TWO: Quantitative Determination of Methoxypyrazines in Water and Wines

In this part, a method of determining methoxypyrazines in water and wines is described which involves the use of synthesized isotope-labelled pyrazine compounds as internal standards and solid-phase extraction (SPE) prior to GC/MS analysis. Before analysis of real wine samples, optimization of the solid-phase extraction (SPE) was carried out. It included studies of effects of the presence of ethanol and salt as well as acid on the recoveries of SPE. Significantly, an attempt was made to utilize fractional distillation instead of the conventional steam distillation in preconcentration of analytes prior to SPE.

2.1. Experimental

2.1.1. Instruments

Gas chromatography/mass spectrometric analysis was carried out by a Hewlett Packard HP gas chromatograph 5890 series fitted with a 30m x 0.25mm I. D. fused-silica column with a film thickness of 0.25 µm, coated with HP-5. The splitless injection port was headed to 275 °C. Injection (2µL) of sample in dichloromethane was performed by an automatic sampler. The carrier gas was helium with a pressure of 110 Kpa. For solid-phase extraction, Waters Oasis HLB Extraction cartridges were employed which contained C18 carbon chain-based sorbents designed to have a hydrophilic-lipophilic balance (HLB). Such sorbents were believed to give high and reproducible recoveries for
acidic, basic, and neutral compounds. A vacuum manifold was applied to dry the cartridges after completion of dropwise flow of samples through the SPE cartridges.

2.1.2. Reagents and other materials

The non-deuterated 2-methoxy-3-isopropylpyrazine, 2-methoxy-3-isobutylpyrazine, and 2-methoxy-3-secbutylpyrazine are commercially available from Aldrich and used without further purification. The three deuterated analogues were synthesized in this work. To decrease the impurities likely interfering with analytes, double-distilled water was utilized. Ethanol (85%) was available from Caledon. Sulphuric acid and sodium chloride were purchased from Aldrich. Dichloromethane, used as the introduction solvent for GC/MS, was HPLC-grade available from Caledon. However, in this work GC/MS analysis was carried out to check whether this grade of dichloromethane is pure enough to meet the requirements for existence of allowable quantities of impurity likely interfering with trace organic analysis. Surprisingly, the GC/MS results showed that this commercially available HPLC-grade dichloromethane still contained impurities that could interfere with the analytes of interest because of likely overlapping of the analyte peaks with the solvent impurity peaks. To get rid of the solvent impurities, the HPLC-grade dichloromethane was further purified by fractional distillation. After re-distillation, GC/MS was used to detect the purity of the re-distilled dichloromethane at such time intervals as immediately after fractional distillation, five days later after purification, and one month later after purification. Interestingly, the GC/MS data revealed that some impurities would be absorbed by the re-distilled dichloromethane and the amounts of the
impurities were remarkably increased as the time dichloromethane had been kept since distillation. This should be attributed to the extraordinary capacities of absorption of dichloromethane for organics placed around the solvent. As a result, such behaviour of dichloromethane reminded us of the importance of re-distilling dichloromethane and the necessity of using it as a GC/MS solvent as soon as possible after its purification, especially for a trace organic analysis. In addition, all glassware was meticulously cleaned by washing several times with absolute alcohol and the double-distilled water, followed by oven baking at 200 °C overnight prior to use.

2.1.3. Stock solution and sample preparations

Non-deuterated 2-methoxy-3-isopropylpyrazine stock solution at 50 ppm was prepared by dissolving 5 mg solute in 100 mL double-distilled water. In the same way, the other stock solutions of non-deuterated 2-methoxy-3-isobutylpyrazine and 2-methoxy-3-secbutylpyrazine at 50 ppm were prepared. For deuterated analogues, the stock solutions at 50 ppm were prepared by the same method described above. To assure the accuracy of the quantitative analysis, a concern was expressed over the stability of the stock solutions. Extraction of the stock solutions by SPE, followed by GC/MS analysis, was routinely performed to examine if there could be any changes in the GC/MS chromatograms obtained at every three-month interval since the preparations of the stock solutions concerned. The resultant data indicated that the stock solutions were so stable that the deuterated ones can be absolutely employed as internal standards.
For studies of adsorption of pyrazines on container walls, a series of non-deuterated 2-methoxy-3-isobutylpyrazine samples were prepared at different concentrations: 25 ppb, 50 ppb, 0.5 ppm, 1.25 ppm and 2.5 ppm. Meanwhile, the same amount of deuterated analogue was spiked into the corresponding sample, affording a series of samples with the same amounts of the non-deuterated and deuterated pyrazines. For example, the first sample consisted of 25 ppb non-deuterated 2-methoxy-3-isobutylpyrazine and 25 ppb deuterated analogue. The sample preparation involved taking 5μL non-deuterated stock solution and the same volume of deuterated stock solution with pipette, and then dissolving them in 10 mL double-distilled water.

To investigate the influences of the presence of ethanol on recoveries of SPE, a group of non-deuterated 2-methoxy-3-isobutylpyrazine samples were prepared at different concentrations: 25 ppb, 0.5 ppm, 1.25 ppm and 2.5 ppm. Each sample was then spiked with the same amount of deuterated analogue. For example, one sample contained equivalent amounts of the non-deuterated solute and its deuterated analogue, i.e., all at 25 ppb. Additionally, 1 mL of ethanol was added to generate 10% (v/v) ethanol in 10 mL aqueous solution. To reveal the influence of so-called salting-out on the recoveries of SPE, a series of non-deuterated 2-methoxy-3-isobutylpyrazine samples were prepared at different concentrations: 25 ppb, 0.5 ppm, 1.25 ppm and 2.5 ppm. Each sample was then spiked with the corresponding same amount of deuterated analogue. Finally, 1 g NaCl was added to yield 10% (m/v) NaCl in 10 mL aqueous solutions.

In order to have an insight into the combined effects of both ethanol and the salt on the recoveries of SPE, a series of non-deuterated 2-methoxy-3-isobutylpyrazine samples were prepared at different concentrations: 25 ppb, 0.5 ppm, 1.25 ppm and 2.5 ppm.
Repeatedly, each sample was then spiked with the corresponding same amount of deuterated analogue. Then, 1 mL ethanol and 1 g NaCl were added to give 10% (v/v) ethanol and 10% (m/v) NaCl, respectively in 10 mL aqueous solutions. To shed light on the influence of acid on the recoveries of SPE, a series of samples were prepared in the same manner that was used for studies on the above-mentioned effects on the recoveries. One difference was an addition of 0.1 mL 4 M sulphuric acid to bring these samples to pH 3.0. Moreover, to examine if the use of a so-called vial insert can enhance the sensitivity of the GC/MS, two 100 mL samples were prepared which contained non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine, both at 50 ppt. One is in 100% double-distilled water and another in 10% (v/v) ethanol and 10% (m/v) NaCl aqueous solution.

To make a calibration curve with isotope labelled standards, wine models needed to be made which consisted of ethanol and water, the two major components of wines. Wine model I contained non-deuterated 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine as well as the other two deuterated analogues, all at 0.25 ppb in 100 mL double-distilled water. Then, 10 mL ethanol and 1 mL 4 M sulphuric acid were added to the sample to generate a wine model of 10% (v/v) ethanol in 100 mL aqueous solution at pH 3. For wine model II, the non-deuterated 2-methoxy-3-isobutylpyrazine and 2-methoxy-3-isopropylpyrazine were prepared at 50 ppt each, followed by spiking the other two deuterated analogues at 0.25 ppb each in the solution. Addition of 10 mL ethanol and 1 mL 4 M sulphuric acid gave the wine model II of 10% (v/v) ethanol in 100 mL aqueous solution at pH 3. Wine model III was made by preparing 5 ppt non-deuterated 2-methoxy-3-isobutylpyrazine and 375 ppt non-deuterated 2-methoxy-3-isopropylpyrazine in 100 mL aqueous solution. The resultant solution was spiked with the two
corresponding deuterated analogues both at a concentration of 250 ppt. Next, addition of 10 mL ethanol and 1 mL 4 M sulphuric acid gave rise to the wine model III of 10% ethanol in 100 mL aqueous solution at pH 3. Wine model IV consisted of 10 ppt non-deuterated 2-methoxy-3-secbutylpyrazine and 125 ppt non-deuterated 2-methoxy-3-isopropylpyrazine in 100 mL aqueous solution spiked with the other two deuterated analogues both at 250 ppt. Addition of 10 mL ethanol and 1 mL 4 M sulphuric acid to the aqueous solution generated the wine model IV of 10% ethanol in 100 mL aqueous solution at pH 3.

For the wine sample, a special kind of wine was utilized in this work. It was a white wine with 10 beetles per litre. During the period of wine-making, live HA beetles were added to re-hydrated juice in a 20 L glass carboy at rate of 10 beetles per litre of juice. It was hoped to use this kind of wine to relate methoxypyrazines in the wine to the beetles which are believed to be the source of the methoxypyrazines in the wine. In this work, 100 ml wine with 10 beetles/L was spiked with 0.25 ppb of the three isotope-labelled pyrazines: 2-methoxy-3-isopropylpyrazine, 2-methoxy-3-isobutylpyrazine, and 2-methoxy-3-secbutylpyrazine.

2.1.4 Isolation of the analytes

Isolation of the volatiles is a crucial step since only quite a small volume of concentrated sample can be introduced to GC/MS analysis. Our studies were focused on a quantitative determination of the samples at ppb or ppt level. So, a large volume of
sample needed to be treated to deliver a limited volume of sample which was then used for GC/MS analysis.

(1) Liquid-liquid extraction

To examine whether or not the methoxypyrazines of interest could be adsorbed onto the container walls, the empty flask that had previously contained the methoxypyrazine sample was extracted with 3 mL of dichloromethane. The organic phase was dried over anhydrous Na₂SO₄, then filtered through filter paper and concentrated under an argon stream down to 1 mL sample for GC/MS analysis.

(2) Solid-phase extraction (SPE)

Three steps were involved in SPE. In step 1, the SPE cartridge must be conditioned with 2 mL methanol. This conditioning step enables the sorbent materials to be wetted and thus be ready for the sample to pass through. Samples, used for studies of the influences of the presence of ethanol, salt or acid on recoveries of SPE, were loaded on the top of the cartridge in step 2. The interaction between the analytes and the sorbents can be affected by the sample flow rate. In this work, a dropwise flow rate was applied to enhance the retention of the analytes onto the sorbents. In step 3, vacuum was applied to dry the cartridge once no drops flowed down from the cartridge. In the final step, the analytes were eluted from the sorbent of the SPE with 1 mL dichloromethane.
(3) Fractional distillation-SPE technique

In the literature\(^2\), steam distillation was employed to separate and preconcentrate methoxypyrazines for the quantitative determination of the analytes of interest by HPLC. The reason why steam distillation was adopted for the HPLC analysis was because wines usually contain non-volatile phenolic material that would co-elute with the pyrazines under the chromatographic conditions set for the HPLC analysis. However, in our wine analysis by GC/MS fractional distillation was employed instead of steam distillation in the pre-treatment of samples prior to SPE. Replacing steam distillation by fractional distillation is based on such a consideration that fractional distillation is intrinsically simple and has less equipment needed and therefore was examined.

For fractional distillation to be used for the wine model samples and the wine sample, a 250 mL sample flask was utilized to contain 100 mL sample. A 50 mL receiving flask, immersed in an ice bath (0°C) was connected to the sample flask by a water-cooled condenser and an air-cooled fractional column. The sample was distilled for 60 min in a fractional mode under atmospheric pressure. At the end of the 60-min distillation, an approximate 10 mL of distillate was obtained; 10 g NaCl was added and then brought to 100 mL with double-distilled water. Next, the 100 mL sample was passed through a preconditioned Waters C\(_{18}\)-SPE cartridge at a dropwise flow rate. Pyrazines were eluted from the cartridge with 1 mL dichloromethane. The 1 mL sample in dichloromethane was concentrated under an argon stream down to 250 \(\mu\)L and then transferred with pipette to a vial insert. The 250 \(\mu\)L sample in the vial insert was concentrated under an argon stream down further to 100 \(\mu\)L for GC/MS analysis.
2.1.5. GC/MS methods

The GC oven temperature was programmed to provide a 2.20-min delay at 50°C. The temperature of 50°C was held for another 2-min, then followed by a temperature rise of 15°C/min to 200°C. The final temperature of 200°C was held for 4 min. The injection port was heated to 200°C. The interface between the GC and the MS was kept at 280°C. Injection (2 µL) of sample in dichloromethane was performed by automatic sampler.

To maximize the sensitivity of the mass spectrometer as a detector, selected ion monitoring (SIM) mode was applied in this work. For quantification, the mass of the base peak of the analyte was selected. Therefore, the masses 124, 127 and 166 were chosen in SIM to quantitatively determine both non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine in the studies of the influences of the presence of ethanol, salt and acid on the recoveries of the solid-phase extraction. Also, the combination of masses 124, 127 and 166 in SIM was utilized to examine whether or not the adsorption of the pyrazines onto the container walls could happen. Only two masses (124 and 127) were selected to confirm increased sensitivity by using a vial insert. The same two masses examined in the SIM mode, on the basis of their being the base peaks of the non-deuterated and deuterated pyrazines, facilitated the relevant analysis of the water samples at ppt levels.

In the analysis of a real wine sample, three pairs of masses, characteristic of the three non-deuterated methoxypyrazines and their deuterated analogues, were selected in SIM to examine whether or not the pyrazines of interest exist in the wine analysed. To
establish the calibration curves, the corresponding combinations of mass pairs that had been used in the wine analysis were applied to the wine model analysis.

2.2. Results and Discussion

2.2.1. Adsorption of pyrazines on the container walls

Quantitative determination of the analytes at ppt levels becomes a real challenge due to the limitation of detecting ability of the instrument itself. Another reason why ultra-low levels remain undetected might be that the trace analytes could still remain on the surface of the glassware after their seemingly thorough removal from the sample to the cartridge of the solid-phase extraction (SPE). Table 7 shows a series of results obtained by extraction of non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine directly from the container walls. The peak areas were integrated based on the base peaks of m/z = 124 and 127 and the retention time matches: 7.94 min for peak at m/z = 124 and 7.92 min for peak at m/z = 127. It can be estimated that the methoxypyrazines at ppb levels were absorbed onto the glassware since it was found that the values of the base peak areas measured from the container walls for the samples at concentrations of 1.25 ppm and 2.5 ppm were within the same range as those detected from the bulk solutions of the samples at concentrations of 25 ppb and 50 ppb. Although the pyrazines absorbed were undetectable for the samples at concentrations of 25 ppb and 50 ppb, the pyrazines could still be absorbed on the container walls. The failure to detect the pyrazines on the glassware might be due to their concentrations being far below the
Table 7. Base peak areas* in arbitrary unit characteristic of non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine detected on container walls. ND means not detectable.

<table>
<thead>
<tr>
<th></th>
<th>25 ppb</th>
<th>50 ppb</th>
<th>0.5 ppm</th>
<th>1.25 ppm</th>
<th>2.5 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z = 124</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>134</td>
<td>454</td>
</tr>
<tr>
<td>m/z = 127</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>122</td>
<td>317</td>
</tr>
</tbody>
</table>

* These peak areas represent ppb levels.

detection limit of the GC/MS used. Consequently, it can be assumed that a slow equilibration of the pyrazines between the bulk solution and the surface of the glassware, could result in the concentrations of the analytes in the bulk solution and on the glassware below the detection limit of the GC/MS.

2.2.2. Effects of the presence of ethanol on the recoveries of SPE

Since ethanol is a major component of wine, it is absolutely necessary to gain an insight into the influence of ethanol on the recoveries of the solid-phase extraction (SPE). To do so, a comparative study between pure water matrix and 10% (v/v) ethanol matrix would help us make sense of how ethanol exerts an influence on the recoveries using SPE. In addition, a novel method was designed which involved preparing the same concentration of non-deuterated pyrazine and the deuterated analogue within the same sample. The equivalent concentration of the non-deuterated pyrazine and the deuterated analogue within the same sample allowed for a constant ratio of peak areas of the
pyrazines despite a variety of concentrations in a series of samples. Figure 11 demonstrates concentration-dependent changes in peak areas of both non-deuterated and deuterated analogues in pure water samples, and the corresponding peak area ratios of the two base peaks characteristic of the pair of pyrazines. The constancy of the peak area ratio accounts for the accuracy of preparing samples and the instrumental measurements. In other words, spiking a same concentration of deuterated analogue into the non-deuterated pyrazine sample becomes a technique useful in an investigation on the influence of an additive on the recoveries of SPE because of the method’s self-checking function based on the constancy of the peak area ratios.

![Graphs showing peak areas and area ratios](image)

Figure 11. Base peak areas and the corresponding area ratios of the non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine in pure water matrix.

Figure 12 displays the linear relationships of peak areas of masses 124 and 127 versus concentrations and the corresponding peak area ratios in a study of the effects of the presence of ethanol on the recoveries of SPE. The constancy of the peak area ratios fully supports the reliability of the results obtained in this study. From Figures 13 and 14,
10% ethanol matrix, post-SPE: a. m/z=124; b. m/z=127

area ratio (post-SPE, 10% ethanol):
ion 124/127

area ratio vs ppm

Figure 12. Base peak areas and the corresponding area ratios of the non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine in 10% (v/v) ethanol matrix.

it is observed that the recoveries of SPE for samples in 10% (v/v) ethanol matrix were much lower than those for samples in pure water matrix. This could be attributed to a decreased polarity of water in the presence of ethanol. As mentioned earlier, the SPE cartridge used in this work is reversed phase in which non-polar analytes can be retained from the polar matrix flowing through the cartridge. As the polarity of the water matrix

Figure 13. A comparison of the base peak areas of the non-deuterated 2-methoxy-3-isobutylpyrazine between the pure water matrix and 10% (v/v) ethanol matrix.
m/z=127(post-SPE): a. pure water matrix;  
b. 10% ethanol

Figure 14. A comparison of the base peak areas of the deuterated 2-methoxy-3-isobutylpyrazine between the pure water matrix and 10% (v/v) ethanol matrix.

decreases with an addition of ethanol, the ability of the reversed phase SPE sorbents to retain the pyrazines could be challenged by the decreased polarity of the water matrix. This is because the pyrazines would rather still stay in water matrix of a lowered polarity while the sample passes through the SPE cartridge. The dropwise flow rate in 10% (v/v) ethanol aqueous samples was approximately 0.16 mL/min but it was 0.3 mL/min in the pure water matrix.

In addition, it is interesting that no peaks of interest were found representing pyrazines adsorbed onto the container walls in the experiments with the 10% (v/v) ethanol samples at concentrations up to 2.5 ppm. Compared with some adsorption of the pyrazines on the glassware in the experiments with pure water samples, no such
adsorption was found with 10% (v/v) ethanol matrix. The lack of adsorption suggests that pyrazines could be readily retained in 10% (v/v) ethanol matrix because the matrix was a lowered polarity in the presence of ethanol. Furthermore, from the above results with 10% (v/v) ethanol matrix it suggests that the recoveries using SPE for wines could be lower than those of SPE for water samples. This urged us to consider an attempt to increase the recoveries of SPE by the addition of salt, which should have a so-called salting-out effect and should enhance the retention of non-polar analytes onto the sorbents of the SPE cartridge.

2.2.3. Effects of the presence of salt on the recoveries of SPE

The sample preparation was identical to that described in the study of effects of ethanol on the recoveries of SPE. This means that equivalent concentrations of non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine were contained within a same sample of 10% (m/v) NaCl. Figure 15 represents the linear relationships of peak areas of masses 124 and 127 versus concentrations and the corresponding peak area ratios obtained from the samples. As analysed before, the constancy of the peak area ratios
Figure 15. Base peak areas and the corresponding area ratios of the non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine in 10% (m/v) NaCl aqueous matrix.

validated the experimental results shown in Figure 15 because reproducibility is reflected by such constancy of the peak area ratios. Figures 16 and 17 supply the comparisons of peak areas of the base peaks characteristic of non-deuterated and deuterated 2-methoxy-3-isobutylpyrazine, which show that the recoveries from SPE decreased when NaCl was added to the system. Generally speaking, the so-called salting-out effect would

![Graph showing m/z=124 (post-SPE): a. pure water matrix; b. 10% NaCl](image)

Figure 16. A comparison of the base peak areas of the non-deuterated 2-methoxy-3-isobutylpyrazine between the pure water matrix and 10% (m/v) NaCl aqueous matrix.
Figure 17. A comparison of the base peak areas of the deuterated 2-methoxy-3-isobutylpyrazine between the pure water matrix and 10% (m/v) NaCl aqueous matrix.

increase the recoveries of SPE since the salt would increase the polarity of the aqueous matrix and then the increased polarity could drive the non-polar analytes onto the sorbents of SPE cartridge. However, our results stand inconsistent with the trend in that the salting-out effect should favour an increase in recoveries by SPE. Such a phenomenon could be interpreted as follows. Prior to SPE separation, the pyrazines left the bulk solution in part for the headspace and the container walls as soon as NaCl was added to the system. This brought in some loss of the pyrazines, causing a lower yield of SPE relative to the pure water samples. This argument can be supported by the fact that in the literatures the salting-out effect was reported to be used to increase the sensitivity of headspace GC/MS because an addition of salt could enhance the fugacity of the non-polar analytes from the bulk aqueous sample to the headspace of the vial. As a
consequence, our experimental results appear to eliminate the possibility of enhancing recoveries of SPE through addition of salt to an aqueous sample.

2.2.4. Effects of both ethanol and salt on the recoveries of SPE

As discussed above, the recoveries of SPE separations can be decreased by either ethanol or salt. Then, what would happen to the recoveries of SPE isolation if both ethanol and salt co-existed in the samples studied? Figures 18 and 19 illustrate a whole profile of the recoveries from SPE affected by such factors as the presence of either ethanol or NaCl or a combination of ethanol and NaCl. Figure 18 is based on m/z = 124 representing the base peak of non-deuterated 2-methoxy-3-isobutylpyrazine and Figure 19 on m/z = 127, the base peak of the deuterated analogue. From the two figures, it is apparent that the recoveries by SPE in the presence of both ethanol and salt were higher than those in the presence of single ethanol or salt. Significantly, this experimental evidence provides us with a means by which the recoveries of SPE for wines could be enhanced by an addition of 10% (m/v) NaCl.
Figure 18. Comparisons of the base peak areas of the non-deuterated 2-methoxy-3-isobutylpyrazine among the four types of samples: the pure water matrix, 10% (v/v) ethanol matrix, 10% (m/v) NaCl aqueous matrix, and 10% (v/v) ethanol plus 10% (m/v) NaCl matrix.
Figure 19. Comparisons of the base peak areas of the deuterated 2-methoxy-3-isobutylpyrazine among the four types of samples: the pure water matrix, 10% (v/v) ethanol matrix, 10% (m/v) NaCl aqueous matrix, and 10% (v/v) ethanol plus 10% (m/v) NaCl matrix.

From Figures 18 and 19, it also makes clear that ethanol can exert almost the same influence on the recoveries of SPE as the salting-out effect on the recoveries of SPE. As a consequence, for an analysis of a pure water sample, no salting-out effect should be introduced into the systems for the purpose of increasing the recoveries of SPE; while for an analysis of a wine sample such salting-out effect should be utilized in order to improve the recoveries by SPE.
2.2.5. Effects of pH on the recoveries of SPE

It is known that methoxypyrazine can behave like a base because of its nitrogen atoms possessing a lone pair of electrons. In the presence of hydrogen ions, the neutral methoxypyrazine molecules could combine with the hydrogen ions to form positive-charged ions:

\[
\text{N}^+\text{OCD}_3 \quad + \quad \text{H}^+ \quad \rightarrow \quad \text{N}^+\text{OCD}_3
\]

As a reversed phase SPE cartridge was used in this work, so the formation of such positive-charged ions could decrease the affinity of the SPE cartridge for the pyrazines. Figures 20 and 21 display the comparisons of the SPE recoveries between the pure water matrix and the acidic matrix at pH 3, based on m/z = 124 and 127, respectively. It is
Figure 20. A comparison of the base peak areas of the non-deuterated 2-methoxy-3-isobutylpyrazine between the pure water matrix and the acidic matrix at pH 3.

found from the figures that the discrepancy of the recovery between the pure water matrix and the acidic matrix enlarged as concentration of pyrazine increased. Interestingly, the overall trend went with the above prediction in that the SPE recovery would decrease for the acidic matrix compared with that for the pure water matrix. Since our results have indicated that no significant discrepancy in SPE recovery in a range of lower concentrations between the acidic samples and the pure water samples, it can be
m/z=127 (post-SPE): data points a denote pure water matrix; b the pH=3 samples

Figure. 21. A comparison of the base peak areas of the deuterated 2-methoxy-3-isobutylpyrazine between the pure water matrix and the acidic matrix at pH 3.

concluded that in this work there would be no need for an addition of hydroxide ion serving as a counterion of the hydrogen ion when quantitatively analyzing pyrazines at ppt levels in a real wine sample.

2.2.6. Use of vial insert to increase sensitivity of GC/MS measurements

In an attempt to quantitatively determine 2-methoxy-3-isobutylpyrazine at 50 ppt in 100 mL aqueous sample, SIM mode was changed to monitoring just the single base peak at m/z = 124 for 200 msec of dwell time from a usual SIM combination of more than two ions selected and 100 msec of dwell time. Using such a special SIM mode increased the sensitivity of the GC/MS measurements, enabling 50 ppt pyrazine in 100 mL sample to
be detected with signal-to-noise ratio (S/N) of more than 3. As shown in Table 8, using SIM with two masses 124 and 127 monitored for 100 msec of dwell time, only one peak at \( m/z = 124 \) would be detectable. Our previous results have attested that the SPE recoveries for 10% ethanol matrix were much lower than those for pure water matrix.

Table 8. 100 ml 50 ppt 2-methoxy-3-isobutylpyrazine (deuterated and non-deuterated) aqueous samples analyzed using vial insert. A is the peak area in arbitrary unit.

<table>
<thead>
<tr>
<th></th>
<th>SIM: 124</th>
<th>SIM: 124</th>
<th>SIM: 124, 127</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dwell time:</td>
<td>dwell time:</td>
<td>dwell time:</td>
</tr>
<tr>
<td>200 msec</td>
<td>200 msec</td>
<td>100 msec</td>
<td></td>
</tr>
<tr>
<td>100 % water</td>
<td>rt = 7.92 min</td>
<td>only ( m/z = 124 )</td>
<td>rt = 7.92 min</td>
</tr>
<tr>
<td></td>
<td>A = 40.0</td>
<td>A = 64.0</td>
<td></td>
</tr>
<tr>
<td>10% EtOH +</td>
<td>no peaks of</td>
<td>rt = 7.91 min*</td>
<td>( m/z = 124 )</td>
</tr>
<tr>
<td>10% NaCl in water</td>
<td>interest</td>
<td>A = 157.0</td>
<td>rt = 7.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A = 175</td>
<td>A = 142</td>
</tr>
</tbody>
</table>

* use vial insert to take 100 µL sample in vial for GC/MS analysis

Therefore, it is understood that no peak of interest was observed in the chromatograms of 50 ppt 2-methoxy-3-isobutylpyrazine in 10% ethanol sample even containing 10% NaCl (referred to Table 8).

To improve the sensitivity of the GC/MS, a vial insert was introduced to decrease the sample volume for the GC/MS analysis down to 0.1 mL from 1.0 mL, the regular sample volume. As presented in Table 8, due to the use of the vial insert it became possible to get 50 ppt 2-methoxy-3-isobutylpyrazine in 10% ethanol matrix detectable by the GC/MS.
Therefore, this provided us with a means by which the vial insert was used to enhance the sensitivity of the GC/MS analysis for a real wine sample.

2.2.7. Calibration curves and analysis of wine samples

With respect to isolation and preconcentration of the wine samples and the wine models, as discussed earlier, fractional distillation-SPE technique was employed instead of steam distillation-SPE technique which had been utilized for HPLC analysis of wines. Our GC/MS results, after sample preparation by the fractional distillation-SPE, disclosed that no peaks were observed related to those components which have higher boiling points than have the analytes of our interest. This is extremely beneficial to GC column maintenance. In addition, compared with steam distillation, the time spent for fractional distillation was relatively short. Interestingly, the fraction in the fractional distillation was collected at 78.5 °C coincident with the boiling point of ethanol. Thus, the fractional distillation was completed as soon as all ethanol was distilled out and collected in a receiving flask immersed in an ice-water bath. To examine if the pyrazines of interest were still left in the sample flask after fractional distillation, the residual sample in the sample flask was isolated and preconcentrated by SPE and then analysed by GC/MS. The resultant data exhibited no evidence of pyrazines of interest in the residual sample after the fractional distillation. Therefore, it could be assumed that evaporation of ethanol from aqueous phase to gas phase at its boiling point accelerated the phase transition of the pyrazines from the bulk aqueous sample to its vapour state that was then condensed together with the ethanol vapour and collected in the receiving flask. In other words, the...
headspace vapours of pyrazines in the sample flask could be continually removed by the ethanol vapours generated at ethanol's boiling point. So, the ethanol vapours could be regarded as a sort of carrier gas removing the headspace vapours of pyrazines and thus activating a non-equilibrium process in which pyrazines were continually removed from the sample flask to the receiving flask. Such pre-treatment method of samples prior to GC/MS analysis has proved to be remarkably effective in our wine analysis.

GC/MS analysis for the wine samples spiked with known amounts of the three isotope-labelled methoxypyrazines has confirmed the existence of 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-secbutylpyrazine in the wine with 10 beetles per litre on a basis of retention time match. The acquired data showed peak area ratios of $A_{137}/A_{140} = 1.42$ for 2-methoxy-3-isopropylpyrazine, and $A_{138}/A_{141} = 0.74$ for 2-methoxy-3-secbutylpyrazine. Surprisingly, no peak at $m/z = 124$ was found related to 2-methoxy-3-isobutylpyrazine, suggesting that any 2-methoxy-3-isobutylpyrazine in the wine tested was below the detection limit.

In order to quantify 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-secbutylpyrazine in the wine studied, the corresponding calibration curves were established based on the wine models in which the known amounts of the deuterated methoxypyrazines analogues were spiked into the known amounts of the corresponding non-deuterated Aldrich methoxypyrazines. Figures 22 and 23 display the calibration curves for 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-secbutylpyrazine, respectively. The resultant curves were linear with coefficients of determination ($r^2$) of 0.999 and 0.998, respectively. For 2-methoxy-3-isopropylpyrazine, the linear relationship curve is given as follows:
Response ratio = 2.0531 × concentration ratio + 0.3276

For 2-methoxy-3-secbutylpyrazine the corresponding linear relationship is:

Response ratio = 5.3288 × concentration ratio + 1.8315

Since the real wine sample was spiked with 250 ppt isotope-labelled 2-methoxy-3-isopropylpyrazine prior to SPE and GC/MS analysis, a calculation based on the resultant ratio $A_{137}/A_{140} = 1.42$ and the calibration curve in Figure 22 revealed that the wine with 10 beetles per litre contained 138 ppt of 2-methoxy-3-isopropylpyrazine. Moreover, a comparison between the two calibration curves in Figures 22 and 23 revealed that the
Figure 22. Calibration curve for 2-methoxy-3-isobutylpyrazine made by isotope dilution assay.

Figure 23. Calibration curve for 2-methoxy-3-secbutylpyrazine made by isotope dilution assay.
detection limit for 2-methoxy-3-isopropylpyrazine was much higher than that for 2-methoxy-3-secbutylpyrazine; the latter was 5 ppt only. In the analysis of the wine sample, the peak area ratio for 2-methoxy-3-secbutylpyrazine and its deuterated analogue was 0.74 of $A_{138}/A_{141}$. This value was much lower than the peak area response ratio at detection limit (refer to Figure 23), suggesting that much lower than 5 ppt of 2-methoxy-3-secbutylpyrazine could be contained in the wine with 10 beetles per litre.
Conclusions and Future Work

This work successfully developed a route for the synthesis of a library of the isotope-labelled methoxypyrazines: 2-d$_3$-methoxy-3-isopropylpyrazine, 2-d$_3$-methoxy-3-isobutylpyrazine, and 2-d$_3$-methoxy-3-secbutylpyrazine. The synthesized methoxypyrazines were confirmed by comparisons of their NMR, MS and GC/MS data with the relevant data from the corresponding commercially available non-deuterated methoxypyrazine compounds. Furthermore, the importance of this work illustrated the suitability of the solid-phase extraction (SPE) in the sample preparation for the quantitative determination of methoxypyrazines in water and wines.

The study showed that the recovery by SPE for the pyrazines from the water matrix in the presence of 10% ethanol was much lower than that from the pure water matrix, suggesting that a so-called salting-out effect can be employed to enhance the recovery of SPE for the pyrazines from wines. Our research confirmed that an increase in the recovery of SPE for the pyrazines from wines was achieved by an addition of salt. However, such a salting-out effect cannot be applied to the water sample extraction aimed at increasing the SPE recovery. Our studies disclosed that the SPE recovery of the pyrazines from pure water matrix appeared to be adversely affected by the addition of salt than observed in the wine sample. In addition, fractional distillation proved to be an important alternative means of pre-concentrating a sample with a relatively large volume prior to SPE because the fractional distillation can be operated more easily than the conventional steam distillation, especially suitable for GC/MS analysis. Another interesting result in this work uncovered that 2-methoxy-3-secbutylpyrazine exhibits an
extremely low detection limit in GC/MS analysis compared with the other two methoxypyrazines’ detection limits. The reason behind this bizarre phenomenon still remains unknown to us, and so our future work could explore it.

At present, the research in our group is underway to quantitatively determine methoxypyrazines in the so-called control wines that were made under circumstances absolutely without the presence of beetles, hoping to reveal the likely influence of the ladybugs upon the amounts of the methoxypyrazines in the wines. In addition, the method developed in this work, namely fractional distillation-SPE plus isotope dilution-GC-MS, could be utilized in the future work to quantitatively determine the pyrazines at trace levels in real water samples.

References


Appendix

The spectra of $^1$H NMR and EIMS for relevant methoxypyrazines are given in the order below:

2-methoxy-3-isopropylpyrazine (Aldrich) (Figures 24 and 25)
2-d$_3$-methoxy-3-isopropylpyrazine (4) (Figures 26 and 27)

2-methoxy-3-secbutylpyrazine (Aldrich) (Figures 28 and 29)
2-d$_3$-methoxy-3-secbutylpyrazine (5) (Figures 30 and 31)

2-methoxy-3-isobutylpyrazine (Aldrich) (Figures 32 and 33)
2-d$_3$-methoxy-3-isobutylpyrazine (6) (Figures 34 and 35)
Figure 24. $^1$H NMR spectrum of 2-methoxy-3-isopropylpyrazine (reference- Aldrich).
Figure 25. EIMS spectrum of 2-methoxy-3-isopropylpyrazine (reference- Aldrich).
Figure 26. $^1$H NMR spectrum of 2-d$_3$-methoxy-3-isopropylpyrazine (4).
Figure 27. EIMS spectrum of 2-d₃-methoxy-3-isopropylpyrazine (4).
Figure 28. "H NMR spectrum of 2-methoxy-3-secbutylpyrazine (reference-Aldrich).
Initial proton test/Ethyl acetate standard

Current Data Parameters
NAME: xc-2-5
EXPMO: 3
PRODOD: 1

F2 - Acquisition Parameters
Date: 20030729
Time: 10:32
INSTRUM: dp-300
PRD/SHD: 5 mm DNP 1H
PULPROG: za-30
TD: 16364
SOLVENT: CDCl3
NS: 4
GS: 2
SMH: 6172.039 Hz
FIORES: 0.378760 Hz
AD: 1.3271540 sec
AG: 645.1
DE: 81,000 usec
OE: 4.50 usec
TE: 300.0 K
DI: 1.00000000 sec

*************** CHANNEL f1 ***************
NJC1: 1H
P1: 5.20 usec
PL1: -4.80 dB
SF01: 300.1318534 MHz

F2 - Processing parameters
SI: 8192
SF: 300.1300044 MHz
NON: EM
SSB: 0
LB: 0.30 Hz
GB: 0
PC: 1.00

1D NMR plot parameters
CX: 20.00 cm
FSP: 9,000 ppm
F1: 2701.17 Hz
F2P: 0.100 ppm
F2: 30.31 Hz
PpHzM: 0.44500 ppm/cm
HzCM: 133.55705 Hz/cm

Figure 30. $^1$H NMR spectrum of 2-d$_3$-methoxy-3-secbutylpyrazine (5).
Figure 32. $^1$H NMR spectrum of 2-methoxy-3-isobutylpyrazine (reference-Aldrich).
Figure 33. ElMS spectrum of 2-methoxy-3-isobutylpyrazine (reference-Aldrich).
Figure 34. $^1$H NMR spectrum of 2-d$_3$-methoxy-3-isobutylpyrazine (6).