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Determination of 2-methoxy-3-alkylpyrazines in wine by gas chromatography/mass spectrometry

by

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Bestimmung von 2-Methoxy-3-alkylpyrazinen im Wein mittels Gaschromatographie/Massenspektrometrie

Zusammenfassung: Einige 2-Methoxy-3-alkylpyrazine wurden aus einem Sauvignon-blanc-Wein durch kombinierte Destillation bzw. Sammeln der Dampfraumkomponenten und Adsorption an einem Ionenaustauscher zum ersten Mal isoliert. Mittels Gaschromatographie/Massenspektrometrie wurde das 2-Methoxy-3-isobutylpyrazin als Hauptkomponente der Methoxypyrazine eines Sauvignon blanc aus Neuseeland identifiziert. Seine Konzentration konnte mit Hilfe des inneren Standards 2-Methoxy-d₃-3-isobutylpyrazin als 35 ± 2 ppt bestimmt werden. Die beschriebene Methode, deren Bestimmungsgrenze unter der Geruchsschwelle liegt, bedarf nur kleiner Probenmengen (< 60 ml).

Key words: wine, flavour, constituent, analysis, Australia.

Introduction

2-Methoxy-3-alkylpyrazines have been identified as the source of the highly characteristic odours of a number of vegetables, including bell peppers (BUTTERY *et al.* 1969) and peas (MURRAY and WHITFIELD 1975). The threshold of olfactory detection for these compounds is extremely low: for example, 2-methoxy-3-isobutylpyrazine has a threshold of only 2 parts in 10^{12} (parts per trillion, ppt) (BUTTERY *et al.* 1969). The identification and monitoring of such small quantities from natural sources have necessitated the use of sensitive and selective instrumental techniques such as gas chromatography/mass spectrometry (GC/MS).

There have been previous reports of the detection of methoxypyrazines in the juice of the grape cultivars Cabernet Sauvignon (BAYONOVE *et al.* 1975) and Sauvignon blanc (AUGUSTYN *et al.* 1982), and therefore the typical herbaceous varietal characters of wines derived from these cultivars have been attributed to the presence of very low levels of these compounds. To our knowledge, however, there has been no published work to date on the isolation and identification of naturally occurring methoxypyrazines in the wines themselves, despite some rigorous attempts to achieve this goal (SLINGSBY *et al.* 1980).

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In Australia, there is an increasing interest in cool climate wines in which definition of varietal character is emphasised. In the belief that methoxypyrazines at extremely low concentrations may be important flavour components in wine, we sought a strategy for their quantitative determination at levels near their organoleptic thresholds. A procedure has been described very recently for the isolation and quantification of methoxypyrazines in spiked wine, based on separation by high-performance liquid chromatography with ultraviolet detection (HEYMANN *et al.* 1986). The limit of detection of this procedure is reported to be 1.2 parts per billion (10^9), almost three orders of magnitude higher than the lowest organoleptically important concentrations (MAGA and SIZER 1973).

Wine is a far more complex chemical mixture than grape juice (RAPP and MANDERY 1986, and references therein) and therefore, in order to isolate selectively vestigial amounts of any methoxypyrazines naturally present in the wine, we have developed methods of isolation that capitalise both on the volatility and the basicity of the target compounds. Our strategies have the added attraction that they require only minimal chemical manipulation. Of the analytical methods available for identification of the methoxypyrazines, only mass spectrometry can accommodate simultaneously both high sensitivity and high molecular specificity. GC/MS has been employed previously to confirm the presence of 2-methoxy-3-isobutylpyrazine in juice from Sauvignon blanc grapes (AUGUSTYN *et al.* 1982) and to determine low levels of 2-methoxy-3-alkylpyrazines used as warning odours by certain aposematic insects (MOORE and BROWN 1981; ROTHSCHILD *et al.* 1984). Preliminary experiments on spiked wines in our laboratories indicated that improved selectivity in the isolation procedure and molecular analysis was required to cope with the greater chemical complexity of wine. The present communication describes the identification and quantitative determination of 2-methoxy-3-isobutylpyrazine in a sample of commercial wine made from Sauvignon blanc grapes. The wine chosen was a New Zealand 1985 Sauvignon blanc (Selaks) noted for its herbaceous character.

Materials and methods

Glassware was washed either in laboratory detergent (Decon 90) for large-scale equipment or chromic acid followed by sodium bicarbonate solution for small-scale equipment. The glassware was then rinsed several times with distilled water and baked for several hours at 120 °C. Organic contaminants were removed from the glass-distilled water by passage through a column of Porapak Q. Methylene chloride was HPLC grade (Waters) and was purified further by distillation in glass through a Vigreux column. Sodium chloride was AR grade which was heated at 300 °C for several hours before use. Potassium hydroxide was AR grade. Ion-exchange resin (Bio-Rad, AG50WX4 AR) was used as received.

Synthesis of methoxy-alkylpyrazine standards

The unlabelled 2-methoxy-3-alkylpyrazines (alkyl = methyl, isopropyl, *sec*-butyl or isobutyl) were synthesised by the method of MURRAY and WHITFIELD (1975) and their homogeneity checked by GC/MS and NMR spectrometry. The internal standard for quantification of 2-methoxy-3-isobutylpyrazine was the methoxy- d_3 analogue, which was prepared in the following manner. 2-Chloro-3-isobutylpyrazine (MURRAY and WHITFIELD 1975) was refluxed in dry tetrahydrofuran containing sodium methoxide- d_3 (1.5 mole equivalent) prepared from methanol- d_4 (Stohler Isotopic Chemicals, 99 %,

1.5 mole equivalent) and sodium hydride-oil suspension (60 %, 2 mole equivalent) until the reaction was complete (thin layer chromatography, methylene chloride on silica gel). 2-Methoxy- d_3 -3-isobutylpyrazine was isolated by addition of water and extraction into pentane and purified by chromatography on silica gel (elution with pentane to remove mineral oil and 1 : 1 methylene chloride-diethyl ether to elute product), followed by bulb distillation at atmospheric pressure. MR spectrum ($CDCl_3$): δ 0.90, 0.97, d, 6H $(CH_3)_2CHCH_2$; 2.17, m, 1H, $(CH_3)_2CHCH_2$; 2.69, d, 2H, $(CH_3)_2CHCH_2$; 7.97, q, 2H, J_{HH} 2.84 Hz, pyrazine ring protons. λ_{max} 277 nm, ξ_{max} 6520. The absence of the methoxy signal in the NMR spectrum confirmed that the product was > 99 % d_3 -labelled.

Standard solutions of the methoxypyrazines were prepared by weighing the purified synthetic compounds followed by appropriate dilution with methylene chloride. The very dilute standards for GC/MS were prepared as required from the stock solutions, which were stored at $-5^\circ C$ in the dark. Calibration curves were determined from standard mixtures containing 0.505 ng/ μ l of 2-methoxy- d_3 -3-isobutylpyrazine.

Detection of methoxy-alkylpyrazines

Mass spectra were determined on a VG Micromass 70-70F mass spectrometer interfaced to a Hewlett Packard 5790A gas chromatograph and a VG 11-250 data system. The vitreous silica capillary columns used for chromatographic resolution of the mixtures were a bonded phase 5 % phenyl methylsilicone (SGE, BP5, 25 m \times 0.33 mm, 0.5 μ m phase thickness) and a bonded phase Carbowax (SGE, BP20, 25 m \times 0.33 mm, 1.0 μ m phase thickness). The columns were coupled directly to the centre of the ion source by insertion into the vacuum through a heated glass-lined steel jacket. Solutions for analysis were injected directly using a Hewlett Packard 19290B cool on-column injector to avoid molecular discrimination. A retention gap (GROB *et al.* 1985) of deactivated vitreous silica (SGE, 1.0 m) was used to accommodate larger than usual injections (ca. 3–6 μ l) without significant loss of chromatographic resolution. Injections were made at 30 $^\circ C$ followed after 2 min delay by a steep temperature rise (25 $^\circ C$ /min) to 90 $^\circ C$ (BP5) or 110 $^\circ C$ (BP20) and then a shallow temperature gradient (5 $^\circ C$ /min for BP5, 3 $^\circ C$ /min for BP20) to elute the methoxypyrazines. Helium was used as carrier gas at a flow rate of 1 ml/min.

Electron ionisation mass spectra were obtained at an electron energy of 70 eV and a trap current of 100 μ A. Positive-ion chemical ionisation mass spectra were obtained using ammonia as reagent gas at a source pressure of 60 Pa, with an electron energy of 50 eV and a filament emission current of 500 μ A. The source temperature in each case was 180 $^\circ C$.

Detection of the trace quantities of pyrazines eluting from the GC was carried out by selected ion monitoring using the 11-250 data system for accurate control of the instrumental operations and for acquisition and processing of the mass spectral data. Cyclical sampling of the selected mass channels was effected reproducibly by linked switching of the electric sector and accelerating voltages at constant magnetic induction. The selected mass channels in the electron ionisation mode were m/z 124.06, 127.08, 137.07, 138.08, 151.09 and 152.10; while those for the chemical ionisation mode were m/z 153.10, 167.12 and 170.14. Channel dwell times were 50 ms for the former and 100 ms for the latter. Relative ion abundances were assessed from peak areas.

Isolation of methoxy-alkylpyrazines from wine

Two methods were developed for isolating the methoxypyrazines from the wine after addition of the internal standard (2-methoxy- d_3 -3-isobutylpyrazine, 33.2 ng/l),

each method using an ion exchange resin to trap the volatile bases. In the first method, the pH of the wine (200 ml) was adjusted to 4–5 by addition of aqueous KOH (10 %) and the wine distilled at atmospheric pressure till 50 ml of distillate had been collected. Ion exchange resin (200 mg) was added to the distillate and left in contact overnight in the dark. The resin was filtered and washed with water. Water (1 ml) was added and the pH adjusted to 10 by dropwise addition of 10 % KOH solution. After 20 min, the supernatant solution was removed, the resin washed with water (1 ml) and the aqueous solutions combined. The resin was then washed with methylene chloride (1 ml) and the resulting washing used to extract (twice) the aqueous solutions to which sodium chloride (200 mg) had been added. The combined methylene chloride extracts were transferred to a Reacti-vial (Pierce, 1 ml) and allowed to evaporate to about 40 μ l in a gentle updraught of dry nitrogen around the vial at room temperature over 6–7 h. The walls of the vial were washed with the solution, which was transferred to a 100 μ l Reacti-vial, after centrifugation to facilitate recovery of the solution. The 1 ml Reacti-vial was washed a second time by applying 10–15 μ l of methylene chloride to the upper surface, sealing the vial and cooling it in dry ice for a few minutes. After centrifugation, the recovered washings were added to the solution in the 100 μ l vial. The washing was repeated if the odour of methoxypyrazines was still discernible in the 1 ml vial. Evaporation of the solution was allowed to continue until, after washing the walls as described previously, 10–15 μ l remained for assay by GC/MS.

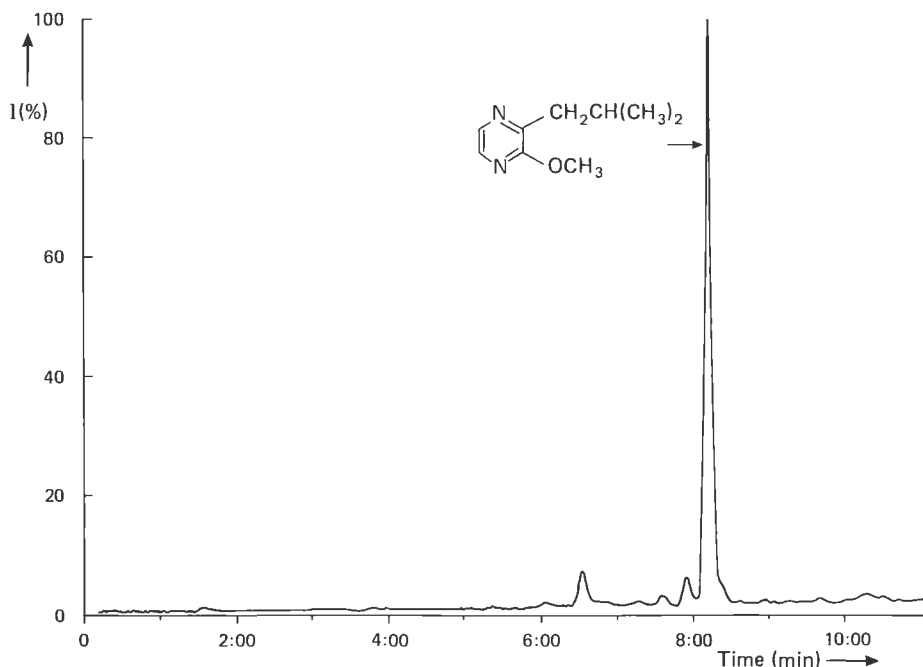
The second method for capturing the methoxypyrazines was a novel headspace technique. High-purity nitrogen at 500 ml/min was bubbled into the wine (300 ml, pH 4–5) at room temperature through a glass sinter and the effluent was filtered through the ion exchange resin (300 mg) contained in a Pasteur pipette. After sparging for 5 h, the trapped bases were recovered from the resin as in the first method. Alternatively for small samples, the final evaporation of the methylene chloride extract from 40 μ l was accomplished within a bent capillary tube by continually wetting its surface with the contents until a volume remained for GC/MS that was sufficient for a single injection (ca. 5 μ l).

Results

The target compounds 2-methoxy-3-methylpyrazine, 2-methoxy-3-isopropylpyrazine, 2-methoxy-3-*sec*-butylpyrazine and 2-methoxy-3-isobutylpyrazine were characterised by selected ion monitoring on two capillary GC columns of contrasting polarity (BP5 and BP20) and under different ionisation conditions (electron ionisation and positive-ion chemical ionisation), using 2-methoxy- d_3 -3-isobutylpyrazine as internal standard. Under the chromatographic conditions used for elution of the methoxypyrazines, the deuterium-labelled internal standard preceded the unlabelled compound by 2–3 s. Calibration curves using chemical ionisation were linear for quantities similar to those in the wine extracts (e. g., $r = 0.99998$, molar ratio 0.1–10.0, isobutyl compound).

Despite the high selectivities of the extraction and detection methods, other unidentified compounds were usually evident in the GC/MS profiles of the wine extracts. However, blank experiments using 10 % ethanolic water for extraction demonstrated that impurities derived from the resin and solvents did not interfere at the calibrated retention times for elution of the target compounds.

The molecular ion of m/z 124.06 for the methyl compound (electron ionisation) was not detectable at the calibrated retention time (BP5 and BP20 columns). The limit of detection ($S/N = 3$) for this compound using electron ionisation was 0.1 ppt and there-



Selected ion monitoring profile (positive-ion chemical ionisation/ammonia, BP20 column, 3 °C/min from 110 °C) displaying the MH^+ ion for 2-methoxy-3-isobutylpyrazine recovered from 60 ml of Sauvignon blanc wine.

Mit Hilfe der ionenselektiven Meßtechnik gewonnenes Testprofil (chemische Ionisation mittels positiver Ammoniumionen; BP20-Säule, Aufheizrate 3 °C/min ab 110 °C); es zeigt das MH^+ -Ion des 2-Methoxy-3-isobutylpyrazin aus 60 ml Sauvignon-blanc-Wein.

fore this possible constituent of relatively high olfactory threshold (MAGA and SIZER 1973) was not investigated further because it was below the level of importance.

For the peak appearing at the calibrated retention time for the isobutyl compound on both BP5 and BP20 columns, the relative contributions to the mass channels monitored under electron ionisation conditions corresponded with those for the synthetic compound. Moreover, the strong signal from the extracts for the expected MH^+ ion under positive-ion chemical ionisation (ammonia) conditions coincided precisely on both GC columns with that for a standard injection of 2-methoxy-3-isobutylpyrazine. The molecular selectivities of the extraction and detection can be judged from the experiment where the procedure was carried out on an initial sample of only 60 ml of wine (Fig.).

Estimated levels of the 3-isobutyl compound in various samples obtained by distillation of the wine were 34.4 ppt on the BP5 column and 33.9 ppt, 34.3 ppt and 38.0 ppt on the BP20 column. These levels were in close agreement with those obtained from the sparging method, where the estimated values were 33.0 ppt and 34.6 ppt (BP20). Thus, the mean concentration (\pm SE) of 2-methoxy-3-isobutylpyrazine in the sampled bottles of wine is 35 (\pm 2) ppt. The excellent agreement between results from the different methods is a good indicator of the suitability of each procedure.

Weak signals were obtained from the wine samples under electron ionisation conditions at the calibrated retention times for the 3-sec-butyl compound but their intensi-

ties were insufficient to allow a confident assessment. Nevertheless, there was a small peak under chemical ionisation conditions (MH^+ , m/z 167.12) eluting from BP5 and BP20 columns at the calibrated retention times for the 3-*sec*-butyl compound. From these chemical ionisation experiments, the maximum level of 2-methoxy-3-*sec*-butylpyrazine was estimated to be 0.5 ppt, slightly below the limit of olfactory detection for this compound (TAKKEN *et al.* 1975).

The evidence for the presence of the 3-isopropyl compound in the wine is less rigorous than for the 3-isobutyl compound because it was not possible to suppress the interference from a closely eluting unidentified compound on the BP20 column. However, there was no apparent interference at the calibrated retention time on the BP5 column and the relative abundances of ions under electron ionisation conditions for the coincident peak from the wine extracts corresponded with those expected for the isopropyl compound. Similarly, there was a peak under chemical ionisation conditions (m/z 153.10, MH^+) at the calibrated retention time. On the basis of the results on the BP5 column, the maximum concentration of 2-methoxy-3-isopropylpyrazine in the samples of wine was 6 ppt, which would be above its organoleptic threshold (MAGA and SIZER 1973).

Discussion

Molecular ions in the electron ionisation mass spectra of the target methoxy-pyrazines are generally of low relative abundance but there is some compensation for the purposes of characterisation of these compounds by selected ion monitoring in that the total ion current for each is concentrated among only a few daughter ions. In contrast, the molecular protonated ions MH^+ in the positive-ion chemical ionisation mass spectra of the methoxy-pyrazines, using ammonia as reagent gas, are of high relative abundances because their gas-phase proton affinities surpass that of ammonia. The stability of the MH^+ ions was used to advantage in quantification of the trace levels by selected ion monitoring, while the selectivity of this mode of chemical ionisation diminished the interference to the mass spectral responses from adventitious impurities of low gas-phase basicity.

The small scale isolation and quantification of 2-methoxy-3-isobutylpyrazine in a Sauvignon blanc wine have been accomplished for the first time. This challenging problem was overcome firstly by selecting only the volatile constituents, by using either distillation or headspace trapping. Secondly, an ion-exchange resin was used to select the bases from the vast majority of volatile flavour components. Although 2-methoxy-3-alkylpyrazines are very weak bases in solution, the strongly acidic (sulphonic acid) resin was effective in trapping them from very dilute aqueous ethanolic solution or from the headspace, and they were subsequently released by basification and solvent extraction. Thirdly, by spontaneous evaporation of the dilute extracts in an inert atmosphere and in subdued light to avoid photodecomposition (HEYMANN *et al.* 1986), the concentration of the methoxy-pyrazines for assay was increased over three orders of magnitude compared to the levels in the wine. The low olfactory threshold for the isobutyl compound facilitates the monitoring of the effectiveness of the isolation procedures.

The GC/MS response to the 2-methoxy- d_3 -3-isobutylpyrazine in the spiked wine after extraction was 5–10 % of that for similar quantities injected directly. Despite these relatively low recoveries, it was highly desirable to use simple isolation procedures that minimised the chance of destroying the target compounds. In any case, the

molecular selectivity of the GC/MS method guarantees a sensitivity of detection below the olfactory threshold. This may assume relevance if methoxypyrazines at subliminal levels exert an influence on wine flavour through interactions with other components.

Summary

2-Methoxy-3-alkylpyrazines have been isolated for the first time from a Sauvignon blanc wine by using a combination of distillation or headspace sampling and trapping by an ion-exchange resin. 2-Methoxy-3-isobutylpyrazine has been identified by gas chromatography/mass spectrometry as the major methoxypyrazine in a New Zealand Sauvignon blanc and, with the aid of 2-methoxy- d_3 -3-isobutylpyrazine as internal standard, its concentration has been determined as 35 ± 2 ppt. The method can be applied to small wine samples (< 60 ml) and detection limits are below the olfactory thresholds.

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Eingegangen am 5. 1. 1987

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