JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation and Identification of 2-Methoxy-3,5-dimethylpyrazine, a Potent Musty Compound from Wine Corks

ROBERT F. SIMPSON, DIMITRA L. CAPONE, AND MARK A. SEFTON*

The Australian Wine Research Institute, P.O. Box 197, Glen Osmond SA 5064, Australia

The compound responsible for a "fungal must" taint evident in industry assessments of wine corks was identified as 2-methoxy-3,5-dimethylpyrazine. The identification was made on the basis of gas chromatography/odor analyses, collection of material using micropreparative techniques, determination of chemical properties of collected material, and comparison by gas chromatography/mass spectrometry with an authentic sample, synthesized from 2-hydroxy-3,5-dimethylpyrazine. 2-Methoxy-3,5-dimethylpyrazine is an extremely potent compound with an unpleasant, musty, moldy aroma and an aroma threshold in a white wine of 2.1 ng/L. While its contribution to the frequency and intensity of cork taint in bottled wine is yet to be established, it has been assessed by some wine industry personnel as second only to 2,4,6-trichloroanisole as a cause of cork taint in Australian wine.

KEYWORDS: Cork taint; 2-methoxy-3,5-dimethylpyrazine; 2-hydroxy-3,5-dimethylpyrazine; 7-methyl-1,6-dioxaspiro[4.5]decane; mustiness; cork; wine

INTRODUCTION

Cork taint in bottled wine adversely affects consumer acceptability and decreases the commercial value of the wine. Most large wineries in Australia conduct "in house" assessments of the occurrence of taint in corks to determine whether they will accept each consignment (*1*).

Although 2,4,6-trichloroanisole (TCA) is considered to be the primary cause of cork taint (*2*−*4*), other compounds identified in wine corks also have been implicated in contributing to taint in some bottled wines (*3*). These compounds include geosmin and 1-octen-3-one (*4*). Consequently, TCA cannot be considered the sole cause of cork taint, and this is supported by observations made by winery personnel and in our laboratory.

The largest of the Australian wine companies routinely conducts assessments of wine corks, and these assessments have led to a relatively comprehensive listing of types of taint observed in natural and agglomerated wine corks (*5*). The causes of some of these taints have not been identified, and some of the taints are considered unlikely to cause a problem in bottled wine because the intensity of the taint is low or the taint is an artifact of the assessment procedure. A taint the winery panelists described as "fungal must" was considered by the panelists to be the second most important form of cork taint after TCA. Fungal must taint was claimed on occasions to be present at an intensity that would make the wine undrinkable and, where this taint was assessed as strong, could lead to rejection of batches of cork.

Duncan and colleagues were the first to recognize fungal must as distinct from taints caused by other, known, musty cork contaminants (*5*). The ability of these winery panelists to distinguish fungal must from other forms of cork taint was confirmed by our gas chromatography/odor (GC/O) assessments of extracts of corks. Such extracts invariably gave a discrete, intensely musty, odorous zone in the GC/aromagram that did not correspond to any previously described compound associated with cork taint. However, no peak could be detected in this region of the chromatogram and the causative agent remained unidentified.

This paper reports the isolation and identification of 2-methoxy-3,5-dimethylpyrazine (**1**, **Figure 1**) as the compound responsible for the fungal must taint, a determination of its aroma threshold in a white wine, and the measurement by stable isotope dilution analysis of its concentration in wine in which contaminated corks were soaked.

MATERIALS AND METHODS

Materials. All compounds were obtained from Sigma-Aldrich and were of the highest purity available. All solvents were Mallinckrodt Nanopure grade and were verified for purity by gas chromatography/mass spectrometry (GC/MS) prior to use. Wine samples were obtained from retail outlets. Samples of 7-methyl-1,6-dioxaspiro[4.5]decane were generously donated by Dr. M. T. Fletcher of the University of Queensland.

NMR Analysis. All NMR analyses were conducted using a Varian Unity Inova spectrometer operating at 600 MHz for proton and 150 MHz for carbon spectra, respectively. Spectra are of deuteriochloroform solutions, and assignments were established by HMQC and HMBC experiments.

Analysis by GC/MS/O. Gas chromatography/mass spectrometry/olfactometry (GC/MS/O) was carried out with a Hewlett-Packard (HP) 6890N gas chromatograph fitted with a Gerstel MPS2 autosampler. The gas chromatograph was coupled to an HP 5973N mass spectrometer and a Gerstel olfactory detector port (ODP2). The Gerstel MPS2 was operated in fast liquid injection mode with a 10 μL syringe (SGE, Melbourne, Australia) fitted.

* To whom correspondence should be addressed. Tel: +61 8 8303 6600. Fax: +61 8 8303 6601. E-mail: mark.sefton@awri.com.au.

10.1021/jf049484z CCC: $27.50
Published on Web 07/27/2004

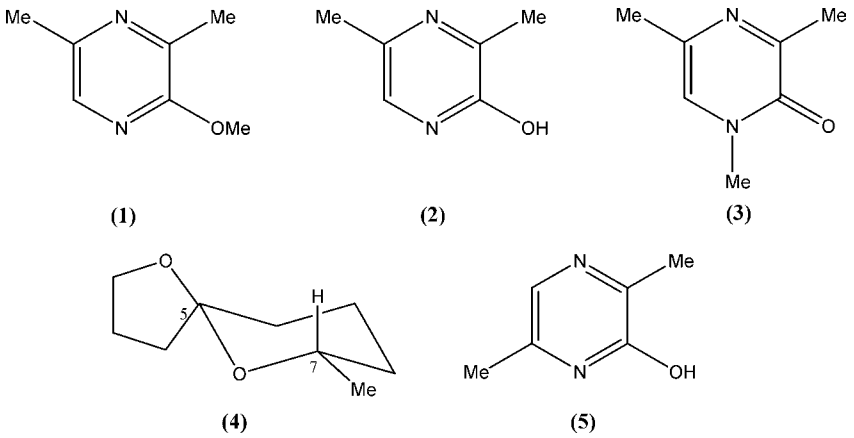

Figure 1. 2-Methoxy-3,5-dimethylpyrazine (**1**), 2-hydroxy-3,5-dimethylpyrazine (**2**), 1,3,5-trimethyl-2(1*H*)-pyrazinone (**3**), (5*S*,7*S*)-7-methyl-1,6-dioxaspiro-[4.5]decane (**4**), and 2-hydroxy-3,6-dimethylpyrazine (**5**).

The gas chromatograph was fitted with either a 30 m × 0.25 mm Phenomenex fused silica capillary column ZB-Wax, 0.25 μm film thickness, or a J&W fused silica capillary column DB-1701 (same dimensions and film thickness). A crosspiece was installed at the end of the analytical column, 2.2 m of 220 μm i.d./320 μm o.d. deactivated fused silica tubing (SGE) was connected from the crosspiece to the ODP, and 2 m of 110 μm i.d./320 μm o.d. deactivated fused silica tubing was connected from the crosspiece to the mass spectrometer, giving a split ratio of 2:1 in favor of the ODP. The carrier gas was helium (BOC Gases, Ultra High Purity) with a flow rate of 1.8 mL/min. A deactivated borosilicate glass liner, 4 mm i.d., with a plug of silanized glass wool (2−4 mm) tapered at the column end was installed in the inlet, and the splitter, at 28:1, was opened after 60 s. Two μL injections were done in pulsed splitless mode with an inlet pressure of 25.0 psi maintained until splitting. The oven temperature was started at 50 °C, held at this temperature for 1 min, increased to 220 °C (to 250 °C for the DB-1701 column) at 10 °C/min, and held at this upper temperature for 10 min. The injector was held at 200 °C (220 °C for the DB-1701 column), and the transfer line at 250 °C (275 °C for the DB-1701 column). Positive ion electron impact spectra at 70 eV were recorded in the range *m*/*z* 35−350 (cycle time, 1.01 s). For determination of **1**, analyses were carried out as described above, except that the GC column (DB-1701) was connected to the mass spectrometer only, the mass spectrometer was operated in the selected ion monitoring (SIM) mode, and the helium flow rate was 1.2 mL/min. The oven temperature was started at 50 °C, held at this temperature for 1 min, increased to 240 °C at 10 °C/min, and held at this upper temperature for 10 min. The injector was held at 200 °C, and the transfer line at 240 °C. Chiral analyses were carried out using the conditions reported previously (*6*).

Syntheses. *2-Hydroxy-3,5-dimethylpyrazine* (**2**) was prepared from the condensation of alaninamide with methylglyoxal according to the method of Jones (*7*), modified to suit present-day laboratory equipment and techniques. Alaninamide was generated from the commercially available hydrochloride by the addition of sodium hydroxide (15 M) in the first step of the preparation. Sufficient alkali was added to give a pH > 7, which was monitored by moistened universal indicator strips. Compound **2** was purified by dissolving the crude product in 10% v/v methanol in chloroform and pouring the solution through a 30 mm × 20 mm i.d. column of Woelm neutral alumina, activity 2. The column was eluted with additional solvent, and the product, after removal of solvent under a vacuum, was purified by crystallization as described by Jones (*7*).

Compound **2** (1.0 g) was dissolved in a small volume of methanol (about 30 mL), diethyl ether was added, and the solution was cooled in ice. An excess of diazomethane in ether was added (*caution*, see safety notes below!). The reaction was complete after 16 h at room temperature; that is, no starting material remained. GC/MS analysis showed only two products in the ratio of 40:60, based on peak area. After quenching the excess diazomethane with acetic acid, the bulk of the ether was removed under a vacuum, water (30 mL) was added,

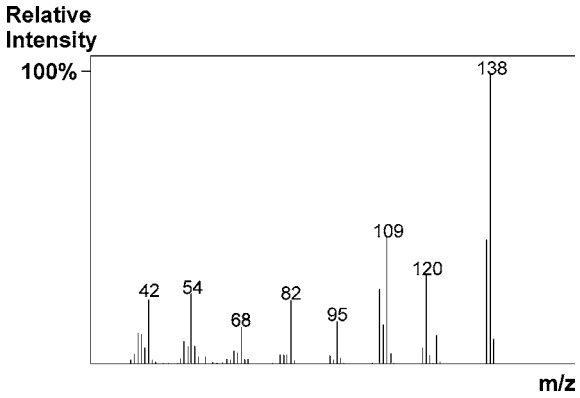

Figure 2. Mass spectrum of synthetic 2-methoxy-3,5-dimethylpyrazine (**1**).

and the solution was extracted with pentane (3 × 20 mL). The solvent was removed, and the product, *2-methoxy-3,5-dimethylpyrazine* (**1**, ca. 40% yield), was purified by microdistillation using a Kugelrohr apparatus. ^{1}H NMR 2.40 (3H, d, J = 0.5 Hz, C_5-CH_3), 2.43 (3H, d, J = 0.5 Hz, C_3-CH_3), 3.92 (3H, s, OCH_3), 7.75 (1H, br. s, C_6-H); ^{13}C NMR 19.1 (C_3-CH_3), 20.1 (C_5-CH_3), 53.4 (OCH_3), 136.7 (C_1), 143.2 (C_5), 143.6 (C_3), 157.1 (C_2); *m*/*z* 138 (100), 137 (43), 123 (10), 120 (31), 109 (47), 107 (27), 95 (16), 82 (34), 68 (16), 54 (35), 52 (11), 42 (38), 39 (16). The mass spectrum (**Figure 2**) was essentially the same as that reported by Mottram et al. (*8*), and the ^{1}H NMR spectrum was virtually identical to that reported by Czerny and Grosch (*9*). The purity of **1** was greater than 99%, based on GC/MS analysis. The aqueous solution was further extracted with dichloromethane (3 × 20 mL), and the solvent was evaporated, yielding 1,3,5-trimethyl-2(1*H*)-pyrazinone **(3**, ca. 60% yield) as a white crystalline solid. ^{1}H NMR 2.13 (3H, d, J = 0.5 Hz, C_5-CH_3), 2.39 (3H, s, C_3-CH_3), 3.41 (3H, s, NCH_3), 6.76 (1H, s, C_6-H); ^{13}C NMR 19.5 (C_5-CH_3), 20.75 (C_3-CH_3), 37.0 (NCH_3), 124.6 (C_6), 130.4(C_3), 155.7 (C_2), 156.9 (C_5); *m*/*z* 138 (91), 110 (41), 109 (100), 95 (30), 69 (11), 68 (68), 54 (11), 42 (51), 41 (19), 39 (15). Kovats GC retention index 2083 (ZB-Wax).

[$^2H_3$]-2-Methoxy-3,5-dimethylpyrazine (**d-1**) was prepared from **1** according to the procedure of Kotseridis et al. (*10*): *m*/*z* 142 (21), 141 (100), 140 (46), 123 (10), 122 (18), 112 (47), 110 (24), 82 (24), 58 (10), 55 (10), 54 (35), 45 (34), 43 (12), 42 (11), 39 (13).

Enrichment of 2-Methoxy-3,5-dimethylpyrazine (1) from Corks. Wine corks (140, 24 mm × 20 mm diameter) that had been classified in the Southcorp Wines assessments as having a moderate incidence of fungal must taint were placed in a 5 L flask with water (1.2 L) purified through a Milli-Q purification system (Millipore Corp., Bedford, MA). Steam distillate (200 mL) was collected and extracted with pentane (2 × 50 mL). The pentane extract was washed with saturated sodium hydrogen carbonate and water, dried over anhydrous

sodium sulfate, and concentrated to ca. 100 μL using a Vigreux column packed with glass Fenske helices. Isolation of a basic fraction of the cork components was obtained in a similar manner, except that sodium tetraborate (36 g) was added prior to distillation to give a pH of 8−9. Sodium hydroxide (5 M) was added to the distillate to give pH 12, and the basified distillate was extracted with pentane (2 × 50 mL). The pentane solution was then extracted with sulfuric acid (5 M, 2 × 10 mL); the acid extract was basified with sodium hydroxide and then re-extracted with pentane. This pentane extract was washed with saturated sodium hydrogen carbonate, dried over anhydrous sodium sulfate, and concentrated to ca. 100 μL with a Vigreux column.

Microscale Preparative GC. The components of the concentrated pentane extract of the steam distillate were chromatographed on a DB-1701 GC column. Those components corresponding to a section of the chromatogram (7−8 min) in which the compound giving fungal must taint eluted (odor detected at 7.53 min) were trapped using a 700 mm length of 0.25 mm deactivated fused silica tubing ("collection loop") connected by a glass connector (SGE) to the tubing normally leading to the ODP. A second glass connector was placed at the exit end of the collection loop so that the loop could be sealed after each collection and placed in a freezer at −18 °C between collections. The tubing was shaped into a coil that was held in place with a cotton thread with slip-knots so that the coils could be released after the final collection. The collection loop was placed in liquid nitrogen a few seconds before connecting to the olfactory detector outlet before each collection. After 12 collections, the exit end of the collection loop was pushed into the inside of a disposable glass capillary tube containing chloroform (5−10 μL). The chloroform was taken up by the capillary tubing (due to capillary action), and the end of the tubing was then lifted so that the solvent slowly flowed under gravity through the collection loop. The other end of the collection loop ("entry end") was placed in a vial insert, and the residual solvent in the collection loop was expelled by gentle pressure. The chloroform solution was partially evaporated by taking the solvent up in a 5 μL syringe and expelling several times. The solution was then injected into the GC/MS system and analyzed using a ZB-Wax column.

Chemical Tests. Material was trapped (7−8 min) from a single injection of the concentrated pentane extract of the steam distillate, described in the preceding section, using the DB-1701 column. In this case, the effluent from the tubing normally connected to the ODP was bubbled through water (500 μL) contained in a 2.5 mL glass vial. A small volume (4 drops, ca. 75 μL) of this solution was added to 1 mL vials, each containing one of the following (1 drop): 30% phosphoric acid, KOH (1 M), sodium borohydride (5 mg) in KOH (1 M), copper sulfate (0.3 M), and 2,4-dinitrophenylhydrazine (DNP, 0.05 M) in 30% perchloric acid. The aroma of the solutions was then evaluated by four assessors familiar with the fungal must taint.

Soaking of Corks in Wine. Wine corks that had been classified as having a sporadic incidence of fungal must taint were obtained from Southcorp Wines. A random sample of 100 corks was treated as follows. Each cork was placed in a 150 mL glass jar, a neutral, white wine (100 mL) was added, and an end of the cork was pierced with a hypodermic needle that submerged the cork in the wine when the glass lid was placed on the jar. The cork was held in the wine for 48 h at room temperature (ca. 25 °C). Several wines were selected that were judged as exhibiting some fungal must taint.

Analysis of Corks and Wine. Sodium tetraborate (3 g) was added to the wine (100 mL) and dissolved by stirring. The wine was transferred to a 150 mL separatory funnel, and internal standard (10 ng of **d-1** in ethanol, 1 mL) was added. The wine was extracted with minimal shaking with pentane (2 × 10 mL). The slight emulsion that formed was broken by adding a few drops of ethanol to the contents of the separatory funnel. The pentane extract was washed with water (5 mL) and then extracted with cold sulfuric acid (1 M, 2 × 10 mL). The flask containing the acid was kept cold by storing in ice. The acid extract was washed with pentane (5 mL) and then basified by the careful addition of saturated sodium hydrogen carbonate (40 mL). The pH of the solution was checked with universal indicator strips to ensure the final pH was greater than 7. The basified solution was extracted with pentane (2 × 8 mL). The pentane extract was washed with water (5 mL) and dried over anhydrous $MgSO_4$. Isooctane (2 drops, ca. 30 μL) was added, and the pentane extract was concentrated using a Vigreux column to ca. 100 μL. The concentrated extract was then transferred to a vial insert, sealed in a 1 mL sample vial, and analyzed by GC/MS in SIM mode. The ions monitored in SIM runs were *m/z* 112, 122, *140*, and 141 for **d-1** and 109, 120, 137, and *138* for **1**. Selected fragment ions were monitored for 30 ms each. The italicized ion for each compound was the ion typically used for quantitation, having the best signal-to-noise ratio and the least interference from other wine components. The other ions were used as qualifiers. The analytical method was validated by a series of standard additions of **1** (0, 2 to 100 ng/L, $n = 6$ for the analyte) to a dry white wine (11.5% ethanol, pH 3.2). The standard addition curves obtained were linear throughout the concentration range, with a coefficient of determination (r^2) of 0.998 and the linear regression equation $y = 1.68x - 0.0105$.

For the analysis of **1** in cork, the cork was ground in a coffee blender and the ground cork was placed in a 100 mL glass jar with a glass stopper. Pentane (40 mL) was added, and the cork was left to soak for 24 h at room temperature (ca. 25 °C). The pentane was decanted through a glass funnel containing a plug of silanized glass wool to remove any cork fragments from the solution. The procedure was repeated, and the combined pentane extract was dried over anhydrous $MgSO_4$. The pentane extract was transferred to a 100 mL separatory funnel, internal standard (10 ng of **d-1** in ethanol, 1 mL) was added, and the pentane was extracted with cold sulfuric acid (2 × 10 mL) and so forth, following the same procedure as for the wine.

Sensory Assessment. The aroma threshold of **1** in a young (<12 months old), neutral, dry white wine was determined according to the American Society for Testing and Materials (ASTM) method E 679-79, using 33 judges, as described by Meilgaard et al. (*11*). The judges were of European origin, aged between 20 and 55, with similar numbers of males and females. The white wine (11.5% v/v, pH 3.2) had a free and total sulfur dioxide content of 35 and 215 mg/L, respectively. Wines were presented in ascending order of concentration of **1**, at 0.3, 0.9, 2.7, 8.1, 24.3, and 72.9 ng/L. Panelists assessed the aroma but did not taste the samples. Those who could detect the spiked wines at all of these concentrations were then tested at lower concentrations of **1**. Panelists were also asked to describe the aroma associated with the wine. The best-estimate threshold for each panelist was the geometric mean of the highest concentration missed and the next higher concentration tested. The group threshold was calculated as the geometric mean of the individual best-estimate thresholds.

RESULTS AND DISCUSSION

Isolation and Identification of the Fungal Must Taint Compound. In our investigations, GC/O assessments of pentane extracts of whole single corks on both a relatively nonpolar and a polar column indicated that the taint was associated with only one part of the chromatogram (although no peak was evident at this retention time), suggesting that the aroma was due to a single component. The observation of this odorous component by GC/O in the room-temperature pentane extracts of corks showed that it was not just an artifact of the distillation process described below.

Steam distillation was chosen as the first step in the isolation and concentration process to reduce the quantity of silicone and paraffin components originating from the cork coatings. Pentane extracts of the steam distillate were then analyzed by GC/MS/O.

The fungal must taint eluted on a Carbowax column at approximately the same retention time as (*E*)-2-octenal and 1-octen-3-ol. However, authentic reference compounds at concentrations similar to those in the extracts did not have a significant aroma and their aroma at higher concentration did not resemble fungal must.

The taint compound eluted at approximately the same time as (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane on a DB-1701 column. Both (*E*)- and (*Z*)-7-methyl-1,6-dioxaspiro[4.5]decane were detected in the cork distillates but had weak aroma only,

at the concentration found in the extracts. The absolute stereochemistry of these compounds was determined by chiral GC/MS analysis of the cork extract and of authentic reference compounds. The dominant isomer was the 5*S*,7*S* form (**4,** 86%), and this was accompanied by minor amounts of the 5*R*,7*R* enantiomer of **4** (12%) and the 5*R*,7*S* diastereoisomer (2%). To our knowledge, these compounds have not been reported previously as components of cork bark. They have been identified in the bark of certain angiosperm trees (*12*), and they are important in the insect communication of several conifer-ophagous bark beetles (*13*). The bark beetles are able to distinguish between host and nonhost trees by the presence of these compounds, and also the males of certain species of beetle synthesize these compounds which are used to repel rival males.

Fungal must material was trapped in water using the GC−ODP tubing and the chemical properties determined, based on the effect on aroma. Mustiness was retained in the presence of potassium hydroxide, sodium borohydride, and copper sulfate but was lost on addition to phosphoric acid. These tests indicated that the compound was basic and was not an aldehyde, ketone, or thiol.

Knowing that the fungal must compound was likely to be basic, the acid soluble components were isolated from a second steam distillation of 140 contaminated corks. This approach provided the means for removal of the acidic and most of the neutral components and gave considerable concentration of the fungal must compound. A significant peak with a retention time corresponding to the fungal must taint was obtained on the ZB-Wax column. The peak contained only one component whose MS and retention time matched those of synthetic 2-methoxy-3,5-dimethylpyrazine (**1**). Compound **1** had a Kovat's retention index of 1417 on the DB-1701 and 1446 on the ZB-Wax column. As reported by Mottram et al. (*8*) in their investigation of an off-odor in machine cutting emulsion, only one isomer of methoxydimethylpyrazine was detected in our investigations of cork volatiles. The two isomers coeluted on the ZB-Wax column but were completely resolved on the DB-1701 column, and as little as 1% of the second isomer (2-methoxy-3,6-dimethylpyrazine) as a proportion of total methoxydimethylpyrazines would have been detected, if present. Compound **1** was also observed when material collected from multiple injections of a pentane extract of the steam distillate of contaminated corks from a DB-1701 column was re-injected onto a Carbowax column. Although **1** overlapped with a similar amount of 5-methyl-2-furfural, a good spectrum of **1** was obtained. The presence of **1** in this pentane extract, prior to partitioning into strong acid solution, showed that it was not an artifact of strong acid treatment.

Synthesis of 2-Methoxy-3,5-dimethylpyrazine (1). Compound **2** was prepared by a modification of the method of Jones (*7*). Three syntheses were carried out during the course of our investigations, and it was found that the yield was strongly influenced by reaction conditions.

When alaninamide was regenerated from its hydrochloride by addition of strong alkali in methanol solution prior to mixing with methylglyoxal, a high yield of **2** was obtained. GC/MS analysis of the reaction product showed the presence of only two components, **2** and 2-hydroxy-3,6-dimethylpyrazine (**5**) (*7*), present in a ratio of 90:10. The addition of water to the reaction mixture (to give a homogeneous solution) gave less **2** (with a ratio of 55:45). When the hydrochloride was used without pretreatment with alkali, the major product was **5** (with a ratio of 5:95) even though the alaninamide hydrochloride and methylglyoxal were quickly added together at low temperature (−20 °C) and alkali was then added within a few minutes. This demonstrates that both the amine and amide nitrogen of the alaninamide can react rapidly with the ketone function of the methylglyoxal and that the regioselectivity of the reaction depends on the availability of the amine nitrogen during the initial few minutes.

Methylation of **2** with diazomethane gave two products. The desired product (**1**) was extracted with pentane after the removal of ether and the addition of water to the reaction mixture and was obtained in near-pure form. It was further purified by microdistillation using a Kugelrohr apparatus to give material that was >99% pure. The major product, 1,3,5-trimethyl-2(1*H*)-pyrazinone (**3**), was obtained from the reaction mixture in near-pure form by further extraction of the aqueous reaction mixture with dichloromethane. The relative proportion of the methylated products **1** and **3** indicates that the starting material (**2**) exists predominantly in the keto-form in the ether−methanol solution to which the diazomethane was added. This is unsurprising since hydroxypyrazines are known to exist as keto−enol tautomers (*14*).

Overall, the preparation of **2** and subsequent methylation with diazomethane provided a sound approach for the synthesis of **1**. Compound **2** was prepared in high yield and was readily purified by recrystallization to give very pure material, free from **5**. This simplified the next step, where there was no need to separate similar components. The methylation step proceeded well to give the required product that was isolated in near-pure form by extraction with a nonpolar solvent, even though it was the minor product. Attempts to convert **2** into **1**, using methyl iodide and bases such as potassium or sodium carbonate, gave low yields and led to considerable decomposition. Other approaches to the synthesis of **1** (*9*) were investigated earlier in our work but found to be less satisfactory.

Aroma Threshold of 2-Methoxy-3,5-dimethylpyrazine (1). The aroma threshold of **1** in a neutral white wine was determined to be 2.1 ng/L. The distribution of best-estimate thresholds is shown in **Figure 3**. The threshold of **1** is unlikely to have been influenced by the possible presence of traces of 2-methoxy-3,6-dimethylpyrazine as Czerny and Grosch (*9*) have shown that the latter has a much higher threshold in water than the former. The most common descriptors used by the assessors were "dirty", "dusty", "musty", and "moldy". A few assessors also described the higher concentrations as "chocolate" or "coffee". The aroma threshold of **1** compares closely with that of TCA, which has an aroma threshold in wine of 1−4 ng/L (*4*, *15*, *16*) and is recognized as one of the most potent taint compounds affecting a wide range of foods and beverages.

Analysis of Cork and Wine in Which the Cork Was Soaked. The internal standard prepared for the analysis of **1** in wine and corks was 2-methoxy-3,5-dimethylpyrazine-d_3 (**d-1**). This material was prepared in high yield by treating **1** with D_2O in the presence of D_2SO_4, using a procedure described by Kotseridis et al. (*10*) for preparing deuterated 2-methoxy-3-isobutylpyrazine. Only three hydrogen atoms in **1** were readily exchanged, and more forcing conditions, such as higher temperature and stronger acid, led to decomposition. Kotseridis et al. (*10*) had indicated that the benzylic protons in 2-methoxy-3-isobutylpyrazine were readily exchanged. The exchange of deuterium for benzylic protons may be promoted in these molecules by the adjacent methoxyl group. No back-exchange of deuterium was observed during the analysis of wines, based on the unaffected isotopic ratio of the major fragment ions *m*/*z* 139−142.

The wine chosen for the analysis of **1** in corks was a "bag-in-box" wine that, therefore, had no contact with a cork prior

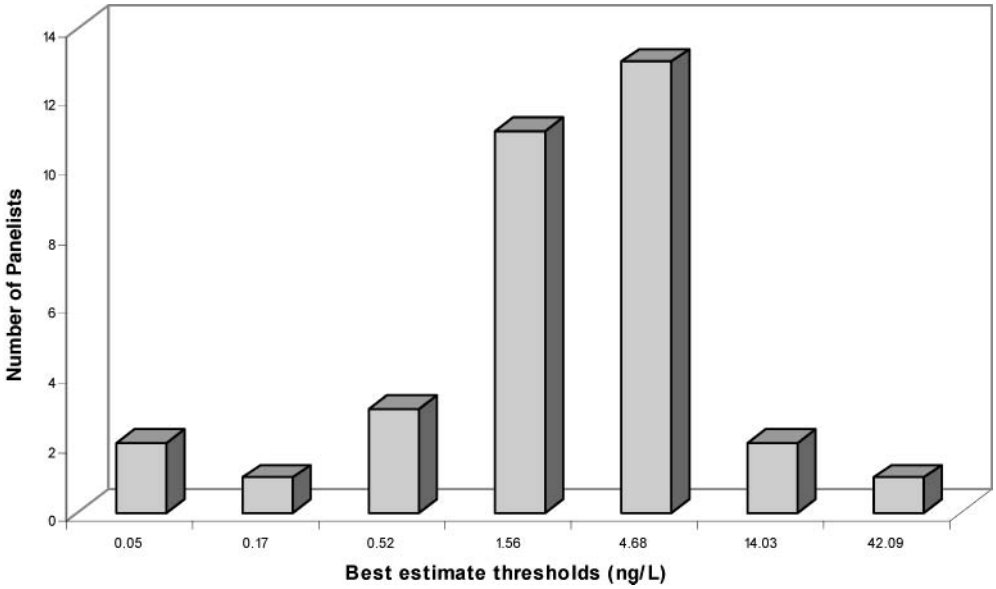

Figure 3. Best-estimate aroma thresholds of 2-methoxy-3,5-dimethylpyrazine (**1**) in a neutral dry white wine.

Table 1. Content of **1** in Corks and Concentration in Wine in Which the Corks Were Soaked[a]

cork no.	content (ng) of **1** remaining in the cork after soaking in wine	concn (ng/L) of **1** in the wine
1	nd[b]	37
2	nd	22
3	52	61
blank (i.e., no cork)		nd

[a] Each cork was soaked in wine (100 mL) for 48 h at room temperature. [b] nd = not detected (limit of detection: 0.5 ng in cork and 1 ng/L in wine).

to the analysis. The content of **1** in each cork and the wine in which the cork was soaked is shown in **Table 1**. Only cork 3 was shown to still contain **1** after soaking, and the associated wine contained the highest concentration of **1**. This wine also had the strongest musty, moldy aroma. The absence of **1** in the blank (i.e., wine only) showed that this compound was cork-derived.

The peaks for the four major ions (*m*/*z* 138, 137, 120, and 109) monitored in the analysis of **1** in the wine extract of cork 3 were symmetrically enhanced on coinjection with authentic **1**, without any change in their ratio, giving further evidence that the compound quantified by selected ion monitoring for these extracts was identified correctly.

Origin and Occurrence of 2-Methoxy-3,5-dimethylpyrazine (1). Mottram et al. (*8*) had reported that **1** was responsible for an obnoxious odor present in certain machine cutting emulsions used in engineering workshops. These authors described the odor as "musty, foul drains, or sour dishcloths". They isolated an aerobic, Gram-negative bacterium which, when grown, gave **1** as the only major component of the volatile fraction isolated from the culture broth. Mottram et al. (*8*) were unable to characterize the organism with known bacterial species but provided a culture to the National Collection of Industrial Bacteria in Aberdeen (accession number 118020). Certain bacteria (*Pseudomonas* spp.) are known to produce pyrazine off-odors, and a mold (*Aspergillus flavus*) produces compounds in the same chemical class (*8* and references therein).

Compound **1** has also been identified in coffee (*9*), where it was described as having an "earthy" aroma. It was shown to be important to the aroma of both raw and roasted coffee and to possess an intense odor; its odor threshold in water was determined as 0.4 ng/L. These authors also suggested that the methoxypyrazines they detected in raw coffee have a bacterial origin.

The origin of **1** in corks was not examined during our investigations. The cutting punches that produce the cork cylinders could be one possible source of the taint. This would explain the apparent total extraction of **1** from two of the corks listed in **Table 1**; that is, the material would be contained on or near the surface of the cork. Also, bacteria capable of producing **1** could be present in areas where the cork is processed or stored and could grow on the cork at some stage of the processing. However, the origin of **1** may not necessarily be bacterial. It has been recognized for many years that certain aroma-intense microbial metabolites occurring in foods and beverages can be produced by different types of microflora. For example, geosmin can be produced by *Actinomycetes* (bacteria) (*17*), cyanobacteria (algae) (*18*), and a number of *Penicillium* spp. (yeasts) (*19*). Davis et al. (*20*) isolated the microflora from wine corks imported into Australia and showed that numerous molds, bacteria, and yeast were present on the corks, but the metabolites produced by these microorganisms were not investigated.

Mottram et al. (*8*) concluded that **1** was likely to be a relatively common cause of off-odor in the environment. However, there has been no further report of **1** as a cause of off-odor in the published literature since then and only one report of its occurrence in a food product (*9*). Perhaps, this is because of the difficulty in analyzing for this compound and the exceedingly low concentration at which it can cause an off-odor. We also think **1** could be a cause of many instances of off-aroma in the environment and in foods and beverages and anticipate that the greater recognition now given to **1** by Czerny and Grosch (*9*) and by this publication and the development of improved techniques to synthesize and identify this compound will lead to more reports of it being a cause of off-odor.

Currently, we have little data on the content of **1** in corks or the extent to which **1** is leached into the wine after bottling. However, indications are that **1** may be the second most important taint affecting wine corks and, like TCA, may be a serious problem for the wine industry by adversely affecting the quality and commercial value of bottled wine.

The presence of **1** in bottled wine as a cause of cork taint is the subject of ongoing investigations at our Institute. In the

meantime, recent work in our laboratory has shown that wine corks have a very low affinity for 3-*iso*-butyl-2-methoxypyrazine (*21*), in stark contrast to their absorptive capacity for TCA (*22*). The greater volatility of **1** (as evidenced by its GC behavior) compared to TCA, coupled with a greater hydrophilicity of the pyrazine, suggests that methods, such as hot water washing or steam cleaning, currently being investigated by cork producers to remove taint from punched corks should have a good chance of reducing or even eliminating this form of cork taint.

SAFETY

Diazomethane is a toxic, explosive gas. It should be handled at all times in a fume hood fitted with a strong exhaust system. The use of a safety shield is also strongly recommended. Users should acquaint themselves with standard chemistry texts on this substance before proceeding with its use.

ACKNOWLEDGMENT

We are indebted to Blair Duncan, formerly of Southcorp Wines, for bringing to our attention this hitherto unrecognized form of cork taint and Alan Pollnitz who conducted the early GC/O assessments of extracts of contaminated corks. We thank Jodie Peters of Southcorp Wines and Andrew Kleinig formerly of Southcorp Wines for providing us with samples of corks and for current observations made during their cork quality assessments. We also thank Mary Fletcher of the Department of Chemistry, University of Queensland, for providing us with samples of the 7-methyl-1,6-dioxaspiro[4.5]decane stereoisomers. We are grateful for the assistance of Gordon Elsey and George Skouroumounis of the AWRI for advice on aspects of the synthetic work, to the staff and students of the AWRI for participating in the sensory panel, and to Peter Høj and Sakkie Pretorius for their continued advice and encouragement.

LITERATURE CITED

(1) Leske, P.; Bruer, N.; Sefton, M. A review of cork sensory assessment methods. In *Proceedings of the ASVO Oenology Seminar on Corks and Closures*; Leske, P A., Eglington, J. M., Eds.; Australian Society of Viticulture and Oenology: Adelaide, 1995; pp 24−26.

(2) Capone, D. L.; Skouroumounis, G. K.; Sefton, M. A. Permeation of 2,4,6-trichloroanisole through cork closures in wine bottles. *Aust. J. Grape Wine Res.* **2002**, *8*, 196−199 and references therein.

(3) Simpson, R. F. Cork taint in wine: a review of the causes. *Aust. New Zealand Wine Ind. J.* **1990**, *5*, 286−288, 289, 291, 293−296.

(4) Amon, J. M.; Vandepeer, J. M.; Simpson, R. F. Compounds responsible for cork taint in wine. *Aust. New Zealand Wine Ind. J.* **1989**, *4*, 62−69.

(5) Duncan, B. Closure quality control at Southcorp Wines. In *Proceedings of the ASVO Oenology Seminar on Corks and Closures*; Leske, P A., Eglington, J. M., Eds.; Australian Society of Viticulture and Oenology: Adelaide, 1995; pp 29−30.

(6) Wilkinson, K. L.; Elsey, G. M.; Prager, R. H.; Tanaka, T.; Sefton, M. A. Precursors to oak lactone II: Synthesis, separation and cleavage of several β-D-glucopyranosides of 3-methyl-4-hydroxyoctanoic acid. *Tetrahedron* **2004**, *60*, 6091−6100.

(7) Jones, R. G. Pyrazines and related compounds. I. A new synthesis of hydroxypyrazines. *J. Am. Chem. Soc.* **1949**, *71*, 78−81.

(8) Mottram, D. S.; Patterson, R. L. S.; Warrilow, E. 2,6-Dimethyl-3-methoxypyrazine: a microbiologically-produced compound with an obnoxious musty odour. *Chem. Ind*. **1984**, 448−449.

(9) Czerny, M.; Grosch, W. Potent odorants of raw Arabica coffee. Their changes during roasting. *J. Agric. Food Chem.* **2000**, *48*, 868−872.

(10) Kotseridis, Y.; Baumes, R.; Skouroumounis, G. K. Synthesis of labeled [$^2H_4$]β-damascenone, [$^2H_2$]2-methoxy-3-isobutylpyrazine, [$^2H_3$]α-ionone, and [$^2H_3$]β-ionone for quantification in grapes, juices and wines. *J. Chromatogr., A* **1998**, 71−78.

(11) Meilgaard, M.; Civille, G. V.; Coerr, B. T. *Sensory evaluation techniques*, 2nd ed.; CRC Press: Boca Raton, FL, 1991; pp 124−133.

(12) Huber, D. P. W.; Gries, R.; Borden, J. H.; Pierce, H. D., Jr. A survey of antennal responses by five species of coniferophagous bark beetles (Coleoptera: Scolytidae) to bark volatiles of six species of angiosperm trees. *Chemoecology* **2000**, *10*, 103−113.

(13) Franke, W.; Kitching, W. Spiroacetals in insects. *Curr. Org. Chem*. **2001**, *5*, 233−251.

(14) Brown, D. J. *Chemistry of Heterocyclic Compounds. Vol. 58*: *The Pyrazines, Supplement I*; John Wiley and Sons: New York, 2002.

(15) Duerr, P. Wine quality evaluation. In *Proceedings of the International Symposium on Cool Climate Viticulture and Enology, Corvallis, OR*; Oregon State University: Corvallis, OR, 1985; pp 257−266.

(16) Liacopoulos, D.; Barker, D.; Howland, P. R.; Alcorso, D. C.; Pollnitz, A. P.; Skouroumounis, G. K.; Pardon, K. H.; McLean, H. J.; Gawel, R.; Sefton, M. A. Chloroanisole taint in wines. In *Proceedings of the Tenth Australian Wine Industry Technical Conference, Sydney, New South Wales, 2−5 August 1998*; Blair, R. J., Sas, A. N., Hayes, P. F., Høj, P. B., Eds.; Australian Wine Industry Technical Conference Inc.: Adelaide, SA, 1999; pp 224−226.

(17) Gerber, N. N.; Lechevalier, H. A. Geosmin, an earthy-smelling substance isolated from Actinomycetes. *Appl. Microbiol*. **1965**, *13*, 935−938.

(18) Safferman, R. S.; Rosen, A. A.; Mashni, C. I.; Morris, M. E. Earthy-smelling substance from a blue-green alga. *Environ. Sci. Technol.* **1967**, *1*, 429−430.

(19) Larsen, T. O.; Frisvad, J. C. Characterization of volatile metabolites from 47 Penicillium taxa. *Mycol. Res.* **1995**, *99*, 1153−1166.

(20) Davis, C. R.; Fleet, G. H.; Lee, T. H. The microflora of wine corks. *Aust. Grapegrower Winemaker* **1981**, *208*, 42, 44.

(21) Capone, D. L.; Sefton, M. A.; Pretorius, I. S.; Høj, P. B. Flavour "scalping" by wine bottle closures. *Aust. New Zealand Wine Ind. J.* **2003**, *18* (5), 16−20.

(22) Capone, D. L.; Skouroumounis, G. K.; Barker, D. A.; McLean, H. J.; Pollnitz, A. P.; Sefton, M. A. Absorption of chloroanisoles from wine by corks and by other materials. *Aust. J. Grape Wine Res.* **1999**, *5*, 91−98.

Received for review March 30, 2004. Revised manuscript received May 27, 2004. Accepted June 6, 2004. This project was supported by Australian grapegrowers and winemakers through their investment body, the Grape and Wine Research and Development Corporation, with matching funds from the Australian Government.

JF049484Z