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# The Effects of Sample Preparation and Gas Chromatograph Injection Techniques on the Accuracy of Measuring Guaiacol, 4-Methylguaiacol and Other Volatile Oak Compounds in Oak Extracts by Stable Isotope Dilution Analyses

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The deuterium-labeled standards [$^2H_3$]-guaiacol and [$^2H_3$]-4-methylguaiacol were synthesized and utilized in a method employing gas chromatography−mass spectrometry to determine the concentration of guaiacol and 4-methylguaiacol in wine or extracts of oak shavings. The method was combined with previously published methods for 4-ethylphenol, 4-ethylguaiacol, *cis*- and *trans*-oak lactone and vanillin, so that all these compounds could be quantified in a single analysis. The method can employ either liquid−liquid extraction or headspace solid-phase microextraction (SPME) and is rapid, robust, precise, and accurate. Under certain conditions, there was artifactual generation, to varying degrees, of guaiacol, 4-methylguaiacol, *cis*-oak lactone, and vanillin during the analysis of oak extracts, especially when diethyl ether extraction and injector block temperatures at or above 225 °C were employed. The most substantial effects were observed for guaiacol, in which results could be exaggerated by over 10 times. These artifacts could be avoided by using headspace SPME or by preparing liquid−liquid extracts with pentane or pentane/diethyl ether (2:1) injected at 200 °C providing spot checks using headspace SPME were performed. Data obtained for previously published quantitative determination of guaiacol in oak extracts should be reexamined carefully, with special attention paid to their respective methods of sample preparation and analysis.

**KEYWORDS: Oak; wine; analysis; artifacts; precursors; guaiacol**

## INTRODUCTION

Although barrels were originally regarded as little more than inert containers, they are now used primarily to enhance wine flavor. Wine is often stored in oak barrels or in contact with oak chips, staves of oak wood, or other oak products, which are all sources of additional wine aroma and flavor components. After the value of the grapes, the cost of oak is the second greatest expense in the production of many wines. It has been estimated that oak costs the Australian wine industry in the range of 89−108 million Australian dollars annually (*1*).

The oak composition and the condition in which wine maturation takes place are of primary importance to the impact of oak on wine quality (e.g., refs 2 and 3). Several aroma compounds identified in wine are derived principally during the process of oak barrel maturation of the wine. Some volatile oak-derived compounds are formed during the charring or toasting of oak barrels, from thermal degradation of lignin or cellulose and hemicellulose (*4*−*10*), and extracted during wine storage.

Hundreds of oak compounds have been identified in the literature, but only several are considered to have an important effect on wine flavor and quality (*8*, *11*−*18*). The relative concentrations of these important compounds, and the effect on wine quality and flavor, due to variations in oak origin, seasoning, coopering, microbial interaction (such as during malolactic fermentation), and other winemaking practices have been studied by many authors (*4*−*5*, *8*, *14*, *16*, *19*−*23*). Seven of the most important flavor compounds in wine, associated with maturation in contact with oak barrels, are guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone, vanillin, 4-ethylphenol, and 4-ethylguaiacol. The last two compounds are products of *Brettanomyces/Dekkera* yeast, which can flourish during barrel maturation. The structures of these seven compounds are shown in **Figure 1**.

Guaiacol (2-methoxyphenol) has a smoky, phenolic, aromatic, burnt, burnt bacon aroma (*19*, *24*−*26*). The aroma threshold of guaiacol in a dry white table wine of unspecified variety has

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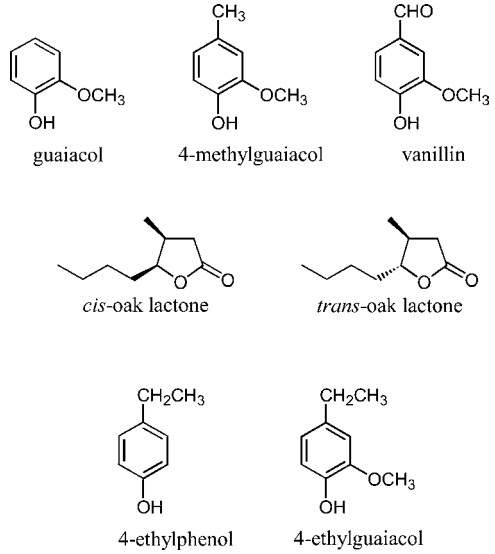


**Figure 1.** Structures of the key compounds discussed in this work.

been reported as 20 μg/L (*27*). Boidron et al. obtained aroma thresholds for guaiacol at 95 μg/L in an Ugni-blanc white wine and 75 μg/L in a Merlot red wine (*19*). 4-Methylguaiacol has a smoky aroma and a reported threshold of 65 μg/L in both the Ugni-blanc and Merlot wines (*19*). In hundreds of analyses done at this laboratory, we have observed that wine matured in oak typically contains between 10 and 100 μg/L of guaiacol and between 1 and 20 μg/L of 4-methylguaiacol, although much higher values have occasionally been determined. Both guaiacol and 4-methylguaiacol are formed by the pyrolysis of lignin (*6*, *9*, *28*) during the toasting of oak barrels, with more guaiacol and 4-methylguaiacol produced at higher temperatures. Chatonnet observed that, of the oak-derived volatile phenols, only these two compounds and vanillin were present in wine at concentrations above their individual aroma thresholds (*14*−*15*, *29*). Above 80 μg/L, Rapp and Versini (*30*) found guaiacol to have a negative effect on wine aroma. In a study of the aroma of barrel aged wine, Spillman (*31*) found that the concentration of guaiacol was negatively correlated with the perceived aroma intensity of "green apple" and positively correlated with "smoky" character in a Chardonnay wine. Other authors have discussed the importance of oak lactone (*32*−*34*), vanillin (*14*−*15*, *29*, *35*), and 4-ethylphenol and 4-ethylguaiacol (*32*, *36*, *37*) to wine flavor.

In any analysis, the internal standard (IS) used should be as similar as possible to the analyte measured, without being present in the matrix to begin with. Isotopically labeled standards are ideal, as they have virtually identical physical and chemical properties to their unlabeled analogues. The principle way to discriminate between the two compounds and measure their concentrations is by mass spectrometry. As the labeled internal standard is virtually identical chemically to the substrate being assayed, the accuracy of the analysis is not reduced by inefficiency in isolation or by analyte decomposition. An advantage of this is that complete extraction of the analyte of interest from the matrix is no longer a necessity. Another advantage is that methods can be streamlined so that several components can be determined in a single extraction and GC/MS run (as done in this paper), even though they have different chemical properties.

The principles, advantages, and applications of SIDA have been discussed before in the literature (*35*, *36*, *38*−*59*). Stable isotope dilution analysis (SIDA) has been used for over a decade to determine the concentration of methoxypyrazines in grapes and in wine (*38*−*45*). Recently, SIDA techniques have been described for the quantitation of trichloroanisole (*46*), vanillin (*35*), ethyl dihydrocinnamate, ethyl cinnamate, methyl anthranilate and ethyl anthranilate (*47*), damascenone, α-ionone and β-ionone (*44*, *48*, *49*, *58*), diacetyl (*50*), oak lactone (*33*), 4-ethylphenol (*36*) and linalool, geraniol, nerol, and α-terpineol (*51*) in wine. In an important paper, Guth (*53*) describes the analysis and odor contribution of 44 volatile compounds in white wine, 41 of which are analyzed using isotopically labeled standards.

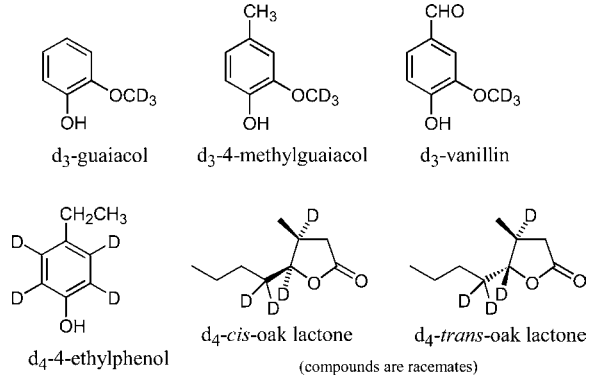


**Figure 2.** Structures of the isotopically labeled standards used.

Even the use of SIDA, however, does not guarantee accuracy of measurement. The generation of an analyte as an artifact of the analytical conditions is one potential source of error, as we have already described for the determination of vanillin (*35*). This paper describes the susceptibility of the analyses of other oak components to such problems and the need for extra caution in determining the concentration of guaiacol in oak extracts.

## MATERIALS AND METHODS

**Materials.** All solvents were Mallinckrodt Nanopure grade and verified for purity by GC/MS prior to use. Unless stated otherwise, all model wine was 10% ethanol in water, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid.

Unlabeled guaiacol and 4-methylguaiacol were purchased from Sigma-Aldrich Australia. Purity of all standards was verified by GC/MS prior to use and periodically prior to making stock standard solutions.

We have already published analytical methods for 4-ethylphenol and 4-ethylguaiacol (*36*), *trans*- and *cis*-oak lactone (*33*), and vanillin (*35*). These publications include details of the syntheses of the internal standards and acquisition of the unlabeled reference materials for these compounds. The structures of the deuterated standards used can be seen in **Figure 2**.

**2-[$^2H_3$]-Methoxyphenol ($d_3$-Guaiacol).** 4-Methyl-2,6-di-*t*-butylphenol (250 mg), followed by 1,2-dihydroxybenzene (catechol) (4.97 g), then [$^2H_3$]-methyl iodide (2.8 mL), was added to a solution of sodium isopropoxide (generated with 2.52 g of sodium hydride) in 2-propanol (320 mL) under nitrogen. The mixture was stirred at 25 °C overnight, then acidified with concentrated aqueous hydrogen chloride to pH < 4 and evaporated to dryness in vacuo. Water (50 mL) was added to the product, which was then extracted with pentane (5 × 50 mL). The combined pentane extracts were dried (sodium sulfate) and the pentane evaporated in vacuo leaving crude product (2.94 g, 54% yield), which was purified by dry column chromatography using acidic alumina (activity IV) as the stationary phase and dichloromethane:pentane (1:1) as the eluant. Column fractions were washed with aqueous sodium bisulfite to remove traces of iodine and dried (sodium sulfate). The solvent was removed in vacuo and the product purified by distillation under reduced pressure with a Kugelrohr microdistillation apparatus to yield $d_3$-guaiacol as clear oil, which crystallized at low temperature (1.62 g, 30% yield, 100% pure by GC/MS). Mass spectrum: *m*/*z* 128

(7%), 127 ($M^+$, 93%), 110 (9%), 109 (100%), 81 (56%), 53 (13%), 52 (10%), 51 (8%), similar to that reported by Cerny and Grosch (*52*).

**2-[$^2H_3$]-Methoxy-4-methylphenol ($d_3$-4-Methylguaiacol).** A solution of [$^2H_3$]-vanillin (500 mg, as made by Spillman et al. (*35*)), potassium hydroxide (1.24 g, 15 molar equiv) and hydrazine hydrate (>99% pure, 0.54 mL, 7.6 molar equiv) in diethylene glycol (20 mL) was heated under nitrogen to 130 °C for 1 h, then 190 °C for 3 h. The solution was cooled, hydrochloric acid (0.3 M, 73.5 mL) was added, and the $d_3$-4-methylguaiacol was isolated with *n*-pentane (6 × 30 mL). The product was purified by distillation under reduced pressure with a Kugelrohr microdistillation apparatus to yield $d_3$-4-methylguaiacol as clear crystals (419 mg, 92% yield, 100% pure by GC/MS). Mass spectrum: *m*/*z* 142 (10%), 141 ($M^+$, 100%), 140 (9%), 123 (77%), 95 (19%), 77 (7%), 67 (9%), 55 (5%). Positive ion electron impact spectra at 70 eV were recorded in the range *m*/*z* 50−350, as described below.

**Quantitative Determination of Guaiacol, 4-Methylguaiacol, 4-Ethylphenol, 4-Ethylguaiacol, *trans* and *cis*-Oak Lactone and Vanillin in One Assay.** *Preparation of Samples for Analysis.* For analysis of liquid−liquid extracts of wine or model wine oak extracts, a solution of [$^2H_3$]-guaiacol (0.500 μg), [$^2H_3$]-4-methylguaiacol (0.500 μg), [$^2H_4$]-4-ethylphenol (2.34 μg), [$^2H_4$]-*trans*-oak lactone (3.36 μg), [$^2H_4$]-*cis*-oak lactone (1.90 μg) and [$^2H_3$]-vanillin (2.50 μg) in ethanol (100 μL) was added to the sample (5 mL) in a screw cap vial using a glass syringe (100 μL Hamilton). The organic solvent (ca. 2 mL) was added, and the mixture was shaken briefly. A portion of the organic layer was then placed in a vial ready for instrumental analysis.

Reference standards containing 100 μL of deuterated internal standards ethanolic solution (as described above) and 100 μL of normal unlabeled analytes ethanolic solution (guaiacol (0.200 μg), 4-methylguaiacol (0.200 μg), *cis*-oak lactone (0.500 μg), *trans*-oak lactone (0.500 μg), and vanillin (0.500 μg)) in diethyl ether/*n*-pentane (1:2(v/v), approximately 1 mL) were used. It is important that these reference calibrants are concocted on the same day and using the same mix of deuterated standards as that used to spike the wine extracts for analysis on that day, with the same glass syringe as that used to add the internal standards to the wine and a separate syringe (preferably of the same type and size) to measure the solution of unlabeled compounds used to make up the reference mix.

For analysis by solid-phase microextraction (SPME), the deuterated standards solution (as described previously) and sodium chloride (ca. 1 g) were added to the wine (5 mL) in a screw cap vial with a Teflon seal. An SPME portable field sampler (Supelco, Bellefonte, PA) fitted with either a 65-μm Carbowax-DVB or a 100 μm PDMS (poly(dimethylsiloxane)) fiber assembly (Supelco) was used to sample the headspace above the stirred wine sample for 20 min at room temperature, immediately prior to instrumental analysis.

*Instrumental Analyses.* Samples were analyzed with either a Hewlett-Packard (HP) 5890A Series II gas chromatograph coupled to an HP 5971 mass spectrometer or with an HP 6890 gas chromatograph coupled to an HP 5973 mass spectrometer. The analytical method was the same for both gas chromatograph/mass spectrometer systems, except that the 6890 gas chromatograph was run in the pulse splitless mode. The gas chromatograph was fitted with an approximately 30-m × 0.25-mm J&W fused silica capillary column DB-1701, 0.25-μm film thickness. The carrier gas was helium (Air Liquide or BOC Gases, high purity). On the 5890A Series II, linear velocity was 31 cm/sec, flow rate was 0.72 mL/min. On the 6890, linear velocity was 50 cm/sec; flow rate was 1.2 mL/min. All flow rates were vacuum compensated at the mass spectrometer interface. The oven temperature was started at 50 °C, held at this temperature for 1 min, increased to 250 °C at 10 °C/min, and held at this temperature for 20 min. The injector temperature was varied (see text), and the transfer line was held at 280 °C. For liquid injections, the sample volume injected was 2 μL. The splitter, at 30:1, was opened after 36 s, and the liner used was resilanised borosilicate glass, tapered, with a plug (2−4 mm) of resilanised glass wool at the column interface. The residence time for the needle in the injector block was approximately 100 ms. Positive ion electron impact spectra at 70 eV were recorded in the range *m*/*z* 50−350 for scan runs.

For quantification of the oak volatiles, mass spectra were recorded in Selective Ion Monitoring (SIM) mode. The ions monitored in SIM runs were *m*/*z* 81, 109, *127* for $d_3$-guaiacol; *m*/*z* 81, 109, *124* for guaiacol; *m*/*z* 95, 123, *141* for $d_3$−4-methylguaiacol; and *m*/*z* 95, 123, *138* for 4-methylguaiacol. MS conditions for the determination of *trans*- and *cis*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*), and vanillin (*35*) are described elsewhere. The italicized ions were the ones used for quantitation (by peak area). Selected ions were monitored for 50 ms each.

The analyses by SPME were done in the same manner, except as follows: the fiber was baked for at least 10 min before retraction; the liner was resilanised borosilicate glass, direct injection (1.5-mm ID); the oven temperature was started at 50 °C, held at this temperature for 1 min, then increased to 250 °C at 10 °C/min, and held at this temperature for 10 min; the injector was held at 220 °C and the transfer line at 280 °C.

*Validation of the Analytical Method.* The precision of the assay was validated by a series of standard addition experiments to red, model, and white wine matrixes. Red and white wines were spiked at 0, 1, 2, 5, 10, 25, 50, and 250 μg/L with guaiacol and 4-methylguaiacol. Another red wine and a model wine were spiked at 0, 1, 2, 5, 10, 25, 50, 250, 500, and 1000 μg/L with guaiacol and 4-methylguaiacol and the other oak volatiles. Validation of the other oak volatiles was as described previously: *trans*- and *cis*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*), and vanillin (*35*).

**Studies on the Artifactual Generation of Oak Volatiles During the Analysis of Extracts of Oak Shavings−Injector and Solvent Effects.** Fine shavings (1-mm thickness) were taken from six oak wood samples, two staves of *Quercus alba*-fine grain (A and B), two of *Quercus alba*-medium grain (A and B), and two of Chestnut Oak (*Q prinus*) (A and B) all supplied by a local supplier in South Australia. The species of the oak samples was assessed by the suppliers, but has not been independently confirmed by us. Approximately half of the shavings from each oak sample were heated in a Carbolite constant temperature (± 1 °C) oven fitted with a Eurotherm digital controller (Medos, Adelaide, Australia) at 175 ± 1 °C for 2 h and then allowed to cool. These shavings were heated in the same Schott bottle in which they were later immersed in model wine. During heating, the bottle openings were wrapped with alfoil, as best as possible, to minimize the loss of any escaping volatiles. The other halves of each set of shavings were the unheated controls. All twelve samples of shavings (ca., 100 g each, but each weighed exactly) were soaked in 1 L of model wine at 25 °C for 1 week, after which time the shavings were removed by filtration through glass wool. Separate 5-mL aliquots of six of the extracts (*Q. alba* fine grain B and Chestnut Oak A and B, heated and unheated) were extracted and analyzed in triplicate by headspace SPME, and also by liquid−liquid extraction with pentane (~2 mL), pentane-diethyl ether (2:1) (~2 mL) and diethyl ether (~2 mL), and each liquid−liquid extract was analyzed by the method at three different injector block temperatures; 200, 225, and 250 °C, in triplicate, giving a total of thirty determinations for each model wine solution of *Q. alba* fine grain B and Chestnut Oak A and B, heated and unheated. Aliquots (5 mL) from the remaining six solutions were extracted and analyzed in triplicate by headspace SPME and also by liquid−liquid extraction with pentane-diethyl ether (2:1) (~2 mL), and the liquid extracts were injected at two different injector block temperatures; 200 and 250 °C, in triplicate, giving a total of nine determinations for each model wine solution of *Q. Alba* fine grain A and *Q. Alba* medium grain A and B, heated and unheated.

At the same time that the extractions and analyses of the twelve oak extracts above were performed, model wines were spiked in triplicate with the unlabeled oak compounds (to concentrations of 200 μg/L guaiacol, 200 μg/L 4-methylguaiacol, 500 μg/L 4-ethylphenol, 500 μg/L 4-ethylguaiacol, 500 μg/L *trans*-oak lactone, 500 μg/L *cis*-oak lactone, and 500 μg/L vanillin) and extracted with 2:1 pentane/diethyl ether, and also with diethyl ether, and analyzed by the method at three injector temperatures, 200, 225, and 250 °C, giving a total of six determinations for each of the three model wine solutions.

## RESULTS AND DISCUSSION

**Quantitative Determination of Guaiacol and 4-Methylguaiacol.** The precision of the multicomponent analysis for

**Table 1.** Correlation Coefficients ($r^2$) of Standard Additions of Guaiacol and 4-methylguaiacol to Red, White, and Model Wine Matrices[a]

| | $r^2$ | | |
|---|---|---|---|
| compound | red wine | white wine | model wine |
| guaiacol | 0.999[b,c] | 1.000[b] | 1.000[b,c] |
| 4-methylguaiacol | 1.000[b,c] | 1.000[b] | 1.000[b,c] |

[a] Validation data for *cis*- and *trans*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*), and vanillin (*35*) have already been published. The data shown in this table is for injections at 200 °C. The same pentane/diethyl ether (2:1) extracts were also run at 220 and 250 °C giving similar results. Thus, there were no injector block artifact generation effects observed for these wine extracts. This is not the case, however, for all extracts of oak shavings as explained in detail elsewhere in this paper. [b] Range 0–250 $\mu$g/L, $n = 8 \times 2$. [c] Range 0–1000 $\mu$g/L, $n = 10 \times 2$.

**Table 2.** Repeatability of the Oak Volatiles Assay at Higher Concentrations[a]

| | amounts measured in $\mu$g/L – septuplicate determinations | | | |
|---|---|---|---|---|
| | spike level | mean | SD[b] | SD/mean |
| | model wine | | | |
| guaiacol | 200 | 204 | 3 | 1.6% |
| 4-methylguaiacol | 200 | 194 | 10 | 5.4% |
| | oaked white wine (1996 Semillon) | | | |
| guaiacol | 200 | 212 | 6 | 2.8% |
| 4-methylguaiacol | 200 | 203 | 6 | 2.9% |
| | oaked red wine (1995 Cabernet Sauvignon/Shiraz/Malbec) | | | |
| guaiacol | 200 | 229 | 6 | 2.5% |
| 4-methylguaiacol | 200 | 201 | 8 | 3.8% |

[a] Model, white, and red wines were spiked and analyzed in septuplicate to show the repeatability of the extraction and analysis. Extracts were all run on the HP 5890 II/5971 GC/MS. Validation data for *cis*- and *trans*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*) and vanillin (*35*) have already been published. The red and white wines give higher values than that added because they contained unlabeled analytes of interest prior to spiking (the wines were made in contact with oak). [b] SD, standard deviation; SD/mean, coefficient of variance, relative standard deviation.

guaiacol and 4-methylguaiacol was validated by duplicate spiked standard additions to red, white, and model wine matrixes. All of the calibration functions obtained were linear throughout the concentration range. **Table 1** shows the correlations for the compounds in model, white, and red wine matrixes. The method was also precise and repeatable, as shown by the data in **Tables 2** and **3**.

Although DB-1701 was the GC column of choice, the method worked equally well on DB-5 and Carbowax columns, although 4-methylguaiacol had similar retention to *trans*-oak lactone on Carbowax, and sometimes coeluted.

The analysis could be performed equally well by headspace SPME. Good results were obtained for both types of fibers trialed (65 $\mu$m Carbowax-DVB and 100 $\mu$m PDMS). Optimum conditions were 100 $\mu$m of PDMS fiber, 1 g of sodium chloride per 5 mL of wine sample, and 20-min extraction time at 25 °C (data not shown).

**Relative Intensity of Mass Spectral Fragments and Ratio Drift.** The relative intensity of mass spectral fragments for fixed concentrations for each internal standard versus its corresponding analyte (e.g., $d_3$-guaiacol vs unlabeled guaiacol) varied slightly (up to ± 10% over 12 months), according to the instrumental operating conditions. It is therefore important to determine the relative molar ion response factors for standard solutions of all compounds under the same instrumental conditions as employed for the analyses of each set of wine samples. Thus, every time a batch of wine or oak extracts was analyzed, replicate reference standards were made containing known amounts of each deuterium labeled internal standard (IS) and each unlabeled analyte. Also, with each batch, a reference standard was run that contained just the deuterium labeled standards portion, to account for ions common to both labeled and unlabeled compounds. This is especially relevant to $d_3$-guaiacol and $d_3$-4-methylguaiacol, as the isotopic labels are the three deuteriums on their methoxy groups. The major cleavage is the loss of this methyl group; hence, the labeled standard and the unlabeled analyte give subsequent fragments of the same mass-to-charge ratio (i.e., guaiacol and $d_3$-guaiacol have the same qualifier ions of *m*/*z* 81 and 109). These qualifier ions can nevertheless be useful in confirming the identity of guaiacol and 4-methylguaiacol in wine and oak extracts, but only the molecular ions can be appropriately used for quantification, unless impractically slow GC programs are used to resolve the labeled IS and unlabeled analyte peaks.

**Table 3.** Repeatability of the Oak Volatiles Assay at Lower Concentrations[a]

| | amounts measured in $\mu$g/L – septuplicate determinations | | | |
|---|---|---|---|---|
| | spike level | mean | SD[b] | SD/mean |
| | model wine | | | |
| guaiacol | 10 | 9.7 | 0.1 | 1.3% |
| 4-methylguaiacol | 10 | 9.7 | 0.2 | 1.7% |
| | bag-in-box dry white wine | | | |
| guaiacol | 10 | 10.6 | 0.1 | 1.3% |
| 4-methylguaiacol | 10 | 9.9 | 0.1 | 1.3% |
| | bag-in-box red wine (Claret) | | | |
| guaiacol | 10 | 14.8 | 0.5 | 3.2% |
| 4-methylguaiacol | 10 | 10.7 | 0.2 | 1.9% |

[a] Model, white and red wines were spiked and analyzed in septuplicate to show the repeatability of the extraction and analysis. Extracts were all run on the HP 5890 II/5971 GC/MS. Validation data for *cis*- and *trans*-oak lactone (33), 4-ethylphenol and 4-ethylguaiacol (36) and vanillin (35) have already been published. The red and white wines gave higher values than that added because they contained unlabeled analytes of interest prior to spiking, indicating that possibly some contact with oak had occurred during winemaking, or that a small amount of guaiacol and 4-methylguaiacol came from the fruit, as guaiacol has been observed in glycoside hydrolysates of red grape juice (72). [b] SD, standard deviation; SD/mean, coefficient of variance, relative standard deviation.

As areas of the extracted chromatograms for (*m*/*z* 124)/(*m*/*z* 81) and (*m*/*z* 127)/(*m*/*z* 81) are virtually identical, that is, (*m*/*z* 124)/(*m*/*z* 81) = (*m*/*z* 127)/(*m*/*z* 81) = $k \Rightarrow$ (*m*/*z*124 + *m*/*z*127)/(*m*/*z* 81) = $k$, this was one of the factors used to ensure peak homogeneity was checked thoroughly.

**Studies on the Artifactual Generation of Oak Volatiles During the Analysis of Oak Extracts−Injector and Solvent Effects.** Guaiacol and 4-methylguaiacol are known to be formed by the pyrolysis of lignin (*6*, *9*, *31*). Therefore, to test the possibility that oak volatiles were released from precursor forms by thermal degradation of soluble lignin in the gas chromatograph injector block during the analysis, duplicate model wine extracts of untoasted and toasted oak shavings were analyzed under a variety of conditions. As controls, model wine (with no contact with oak shavings) was spiked in triplicate with the unlabeled oak compounds of interest and extracted in triplicate for each extraction condition tested, at the same time, the extractions and analyses of the oak extracts were performed. Under all analysis conditions, these controls gave the expected values with < 4% relative standard deviation (RSD).

If the same concentration of an oak volatile is measured, independent of whether the wine is extracted by headspace SPME or liquid/liquid extraction and independent of injector

**Table 4.** Summary–Significant Effects on Guaiacol Determination of Liquid Injection at 200 °C vs SPME[a]

| oak sample | solvent system | guaiacol concn | L/L:SPME[b] | significance[d] |
|---|---|---|---|---|
| Q. Alba fine grain B–raw | 2:1 p/e | 0.23 | 115% | *** |
| Q. Alba fine grain B–raw | ether | 0.29 | 144% | *** |
| chestnut oak A–raw | ether | 0.83 | 107% | *** |
| chestnut oak B–raw | 2:1 p/e | 0.81 | 107% | *** |
| chestnut oak B–raw | ether | 0.86 | 113% | *** |
| Q. Alba fine grain B–heated | ether | 0.33 | 139% | *** |
| *Q. Alba med. grain A–heated*[c] | *2:1 p/e* | *0.11* | *110%* | *** |
| *Q. Alba med. grain B–heated* | *2:1 p/e* | *0.09* | *117%* | *** |
| chestnut oak A–heated | ether | 1.15 | 221% | *** |
| chestnut oak B–heated | pentane | 0.51 | 130% | *** |
| chestnut oak B–heated | ether | 0.69 | 178% | *** |

[a] All guaiacol concentrations are in $\mu$g/g (weight of unheated sample prior to heating) of oak and show the mean of triplicate determinations. Guaiacol was the only analyte that gave significant artifactual formation at 200 °C. Statistically significant increases of less than 5% were also observed for other samples but are not included in the table. [b] L/L:SPME denotes the ratio of the amount of the analyte measured by liquid–liquid extraction (using the solvent system in the second column) over the amount of the analyte measured by SPME, expressed as a percentage. [c] Italics indicate that the oak wood extract was only analyzed by 2:1 pentane/diethyl ether (2:1 p/e) liquid–liquid extraction and SPME. [d] *******, ($p < 0.001$).

**Table 5.** Summary–Significant Effects of Liquid Injection at 225 °C vs SPME[a]

| oak sample | solvent system[b] | analyte | concn | L/L:SPME[c] | significance[d] |
|---|---|---|---|---|---|
| Q. Alba fine grain B–raw | 2:1 p:e | guaiacol | 0.24 | 116% | *** |
| Q. Alba fine grain B–raw | ether | guaiacol | 0.58 | 287% | *** |
| Q. Alba fine grain B–raw | ether | cis-OL | 90.0 | 106% | *** |
| chestnut oak A–raw | 2:1 p:e | guaiacol | 0.85 | 109% | *** |
| chestnut oak A–raw | ether | guaiacol | 1.05 | 135% | *** |
| chestnut oak A–raw | ether | 4-MeG | 0.04 | 150% | *** |
| chestnut oak B–raw | 2:1 p:e | guaiacol | 0.81 | 107% | * |
| chestnut oak B–raw | ether | guaiacol | 1.11 | 147% | *** |
| chestnut oak B–raw | ether | 4-MeG | 0.06 | 136% | *** |
| Q. Alba fine grain B–heated | ether | guaiacol | 0.97 | 315% | *** |
| chestnut oak A–heated | 2:1 p:e | guaiacol | 0.65 | 122% | * |
| chestnut oak A–heated | ether | guaiacol | 2.48 | 467% | *** |
| chestnut oak B–heated | pentane | guaiacol | 0.63 | 164% | ** |
| chestnut oak B–heated | 2:1 p:e | guaiacol | 0.44 | 113% | ** |
| chestnut oak B–heated | ether | guaiacol | 1.25 | 321% | *** |

[a] All concentrations are in $\mu$g/g (weight of unheated sample prior to heating) of oak, and show the mean of triplicate determinations. Statistically significant increases of less than 5% were also observed for other samples, but are not included in the table. [b] 2:1 p/e, 2:1 pentane/diethyl ether; 4-MeG, 4-methylguaiacol; cis-OL, *cis*-oak lactone. [c] L/L:SPME denotes the ratio of the amount of the analyte measured by liquid–liquid extraction (using the solvent system in the second column) over the amount of the analyte measured by SPME, expressed as a percentage. [d] *****, ($p < 0.05$); ******, ($p < 0.01$); *******, ($p < 0.001$).

block temperature, then it can be concluded that artifactual formation of the compound of interest from precursors as a result of the analysis is highly unlikely. This was the case for the spiked model wine controls (with no oak wood extraction) and for some of the real oak extracts, but not all. Results are shown in **Tables 4**−**6**.

The effect of artifactual oak volatile formation varied according to the analyte. The largest effects were observed for guaiacol, with more than 10-fold increases observed when the higher injector temperature (250 °C) and the polar solvent diethyl ether were used (**Figures 3** and **4**). In general, there was much more guaiacol found in the heated wood as compared to the unheated, which is in agreement with published data and our own observations. In contrast to guaiacol, 4-methylguaiacol usually had low 0−6% (but statistically significant) artifact

**Table 6.** Summary–Significant Effects of Liquid Injection at 250 °C vs SPME[a]

| oak sample | solvent system | analyte[b] | concn | L/L:SPME[c] | significance[d] |
|---|---|---|---|---|---|
| *Q. Alba fine grain A–raw*[e] | *2:1 p:e* | *guaiacol* | *0.15* | *242%* | *** |
| Q. Alba fine grain B–raw | 2:1 p:e | guaiacol | 0.26 | 128% | *** |
| Q. Alba fine grain B–raw | 2:1 p:e | cis-OL | 90.0 | 107% | *** |
| Q. Alba fine grain B–raw | ether | guaiacol | 1.55 | 768% | *** |
| Q. Alba fine grain B–raw | ether | 4-MeG | 0.34 | 106% | *** |
| Q. Alba fine grain B–raw | ether | cis-OL | 94.5 | 112% | *** |
| *Q. Alba med. grain A–raw* | *2:1 p:e* | *guaiacol* | *0.17* | *271%* | * |
| *Q. Alba med. grain B–raw* | *2:1 p:e* | *guaiacol* | *0.10* | *193%* | * |
| chestnut oak A– raw | 2:1 p:e | guaiacol | 0.89 | 115% | ** |
| chestnut oak A–raw | ether | guaiacol | 1.77 | 227% | *** |
| chestnut oak A–raw | ether | 4-MeG | 0.05 | 188% | *** |
| chestnut oak B–raw | 2:1 p:e | guaiacol | 0.85 | 110% | *** |
| chestnut oak B–raw | ether | guaiacol | 1.80 | 234% | *** |
| chestnut oak B–raw | ether | 4-MeG | 0.07 | 164% | *** |
| *Q. Alba fine grain A–heated* | *2:1 p:e* | *guaiacol* | *0.14* | *155%* | *** |
| Q. Alba fine grain B–heated | pentane | guaiacol | 0.33 | 108% | *** |
| Q. Alba fine grain B–heated | ether | guaiacol | 2.28 | 750% | *** |
| *Q. Alba med. grain A–heated* | *2:1 p:e* | *guaiacol* | *0.14* | *128%* | *** |
| *Q. Alba med. grain B–heated* | *2:1 p:e* | *guaiacol* | *0.10* | *126%* | *** |
| chestnut oak A–heated | 2:1 p:e | guaiacol | 0.77 | 145% | *** |
| chestnut oak A–heated | ether | guaiacol | 5.89 | 1110% | *** |
| chestnut oak B–heated | pentane | guaiacol | 0.71 | 183% | * |
| chestnut oak B–heated | 2:1 p:e | guaiacol | 0.53 | 137% | ** |
| chestnut oak B–heated | ether | guaiacol | 3.17 | 818% | *** |
| chestnut oak B–heated | ether | 4-MeG | 0.54 | 106% | ** |

[a] All concentrations are in $\mu$g/g (weight of unheated sample prior to heating) of oak, and show the mean of triplicate determinations. Statistically significant increases of less than 5% were also observed for other samples, but are not included in the table. [b] 4-MeG, 4-methylguaiacol; cis-OL, *cis*-oak lactone. [c] L/L: SPME denotes the ratio of the amount of the analyte measured by liquid–liquid extraction (using the solvent system in the second column) over the amount of the analyte measured by SPME, expressed as a percentage. [d] *****, ($p < 0.05$); ******, ($p < 0.01$); *******, ($p < 0.001$). [e] Italics mean that the oak wood extract was only analyzed by 2:1 pentane/diethyl ether (2:1 p/e) and SPME.

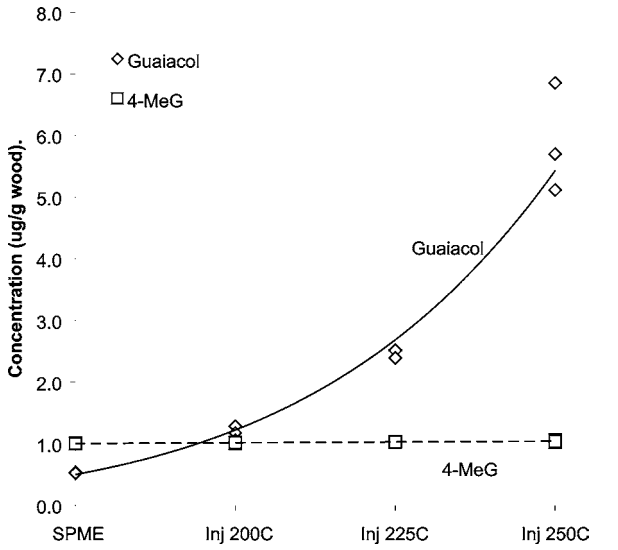


**Figure 3.** Guaiacol and 4-methylguaiacol (4-MeG) concentrations measured in a model wine extract of toasted Chestnut oak–SPME and diethyl ether liquid–liquid extraction injected at different injector block temperatures. All determinations were done in triplicate.

formation when diethyl ether was the extracting solvent at higher injector temperatures.

Highly significant ($P < 0.001$) artifactual generation of guaiacol for diethyl ether extractions of the model wine extract of heated Chestnut Oak, especially at higher injector block temperatures, can be seen in **Figure 3**. The result (0.53 $\mu$g guaiacol/g oak) obtained by headspace SPME can be exag-

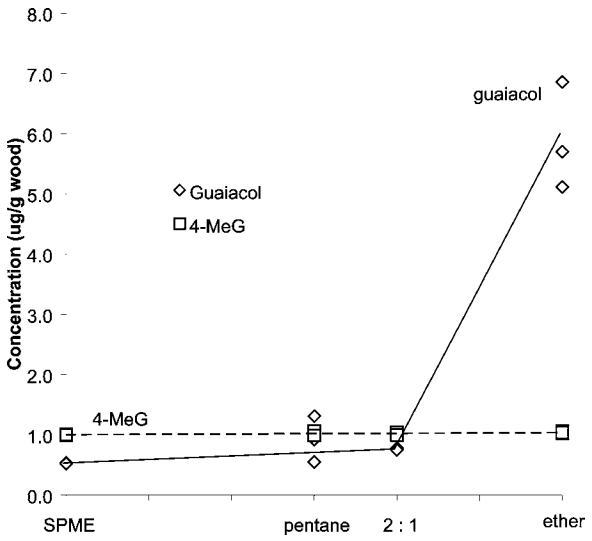


**Figure 4.** Guaiacol and 4-methylguaiacol (4-MeG) concentrations measured in a model wine extract of toasted Chestnut oak–SPME and liquid–liquid extraction with different solvent systems, injection temperature 250 °C. All determinations were done in triplicate.

gerated by more than an order of magnitude when liquid/liquid extracts are analyzed (e.g., mean of 5.89 $\mu$g/g guaiacol, an 1110% factor of increase, whereas 4-methylguaiacol only increased 4% to 1.04 $\mu$g/g mean, for diethyl ether injections at 250 °C). These artifacts could be avoided by using headspace SPME, or minimized in most cases by using liquid−liquid extracts with pentane or pentane/diethyl ether (2:1) injected at 200 °C, providing spot checks using headspace SPME were done.

Note that in **Figure 3**, the precision of the replicates is good for all conditions except the injection at 250 °C. Even for injections at 225 °C, the precision (repeatability) of the analysis is excellent, though the accuracy is poor, with the result exaggerated by more than 450%! It should be emphasized that the accuracy of analyses is commonly presumed from standard curve data, obtained by spiking matrixes with pure samples of analytes. It is clear that had the standards shown in **Figure 3** been analyzed by diethyl ether extraction and injection at 225 °C, a high correlation coefficient ($r^2$) and good precision would have been obtained, giving no indication of the inherent inaccuracy of the method.

As reported previously, although the artifactual formation of *cis*-oak lactone observed was often extremely statistically significant, the total increase was only 0−12% and was highest when more polar solvents (diethyl ether), higher injector temperature (250 °C), and unheated wood were factors (*33*). No 4-ethylphenol and very little 4-ethylguaiacol were formed by heating the oak, and no significant artifact formation of either compound was observed.

Headspace SPME determinations of vanillin generally had relatively poor precision and sensitivity, presumably, due to the relatively low volatility of vanillin and its preference for the liquid phase. Thus quantitative comparisons of vanillin obtained from liquid−liquid extracts with SPME were uninformative. Comparisons of liquid−liquid extracts injected at 200 °C versus injection at 225 and 250 °C demonstrate that in the case of untoasted oak samples a small degree of formation of vanillin in the injector could be observed, with the largest artifactual generation of vanillin being just 7% for an untoasted American oak sample (data not shown). However, when oak extracts are pH adjusted, much greater artifactual generation can be obtained (*35*).

It must be stressed that the results reported here are derived from few American oak samples that were studied in detail. It is not known whether other samples, species, seasoning factors, etc. would increase or decrease the propensity for artifactual generation of guaiacol and other oak volatiles as a result of the analysis. It is entirely possible that much greater artifactual generations of analytes could occur for some samples.

**Alternative Methods for Determination of Guaiacol, 4-Methylguaiacol, 4-Ethylphenol, 4-Ethylguaiacol, Oak Lactone and Vanillin.** In the literature, there is an abundance of analytical methods for the oak volatiles discussed here. For the most part, the determination of these compounds has been carried out in concert with the determination of other oak volatiles. The accuracy of most of these methods, including methods used previously in our laboratory, is open to question. Some authors (e.g., *2*, *60*, *61*) use only one ion for the identification and quantitation of their analytes and internal standards, thus no additional confirmation of identity apart from retention time is possible, and coeluting peaks could remain undetected (*33*). Other authors use flame ionization detection (FID) (*29*, *62*). FID also gives no further confirmation of identity, although some authors use FID to quantitate, but confirm identities via GC/MS (e.g., *4*, *63*). Some authors (e.g. *19*, *21*, *60*, *61*, *63*−*69*) do not determine recoveries, and use inappropriate internal standards, which are quite different in solubility and other chemical properties to their analytes of interest, and yet assume they are extracted at the same rate from the matrix for all the matrixes they analyze. Pérez-Coello et al. (*64*) show one chromatogram with many coeluting peaks and no description of how they estimated areas of these coeluting peaks.

Generally, little or no consideration is given to the possibility that extraction methods could increase the risk of artifact formation during analyses. The relatively polar solvents (e.g., dichloromethane, ethanol/toluene) used by some authors (e.g., *60*, *61*, *63*, *64*, *66*, *69)* could well extract precursors to the volatiles of interest that may then generate those volatiles during the analysis.

Despite earlier reports by us demonstrating the potential for artifact formation as a result of the determination of vanillin (*35*), other authors persist in using conditions conducive to artifact formation, such as high pH (Cutzach et al. (*70*) use initial adjustment of the pH to 8.5 and several washes at pHs from 8.5 to 13!) and inappropriate internal standards (*67*, *68*). Even when higher variance for vanillin is reported (*68)* it is still not attributed to possible problems with the analytical methodology. Boidron et al. (*19*) does not mention any quantitative results for vanillin, despite stating its importance to oak-derived wine flavor. Related papers (*4*, *5*, *29*, *70*) also omit data on vanillin. This implies that at least some of these authors had problems in measuring vanillin.

Some authors demonstrate an awareness that the possibility of artifactual generation of some analytes could be a concern and take measures to avoid potential problems by the use of on-column injection (*53*) or static headspace analysis (*71).* Nevertheless, few papers (*33*, *35*) show experimental evidence that the artifactual generation of oak volatiles actually occurs and the extent to which it can occur under typical extraction techniques, verified by accurate analysis of the same samples under fully robust conditions. In the majority of cases investigated by us, liquid−liquid extraction of oak extracts gives acceptable accuracy (± 5%), provided that factors conducive to artifact formation are minimized. Hence, the extraction should be rapid (minutes) and not involve any adjustment of the pH of

the matrix, a relatively nonpolar solvent system should be used (e.g., 2:1 pentane/diethyl ether), and the injector temperature should be kept to 200 °C. These conditions do not generate artifacts during the analyses of red and white wines that have been aged in contact with oak products (as observed by us performing hundreds of these analyses over several years). However, in model wine extracts of oak shavings, it is still necessary to do "spot checks" with SPME to ensure that no significant artifactual generation is occurring as a result of the analyses.

As demonstrated here, no analysis is foolproof. Even SIDA may yield precise but inaccurate data, and constant vigilance is necessary to eliminate as many potential sources of error as possible.

## ABBREVIATIONS USED

GC, gas chromatography; MS, mass spectrometry; SPME, solid-phase microextraction; SIDA, stable isotope dilution analysis

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