THE ANALYSIS OF VOLATILE WINE COMPONENTS DERIVED FROM OAK PRODUCTS DURING WINEMAKING AND STORAGE

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This thesis is dedicated to the glory of God, Father, Son and Holy Spirit.

"Stop judging by external standards, and judge by true standards."

John 7:24

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Abstract

New methodology was developed for the analysis, by gas chromatography-mass spectrometry, of 2,4,6-trichloroanisole (TCA) and other chloroanisoles in wine and cork, using a polydeuterated form of TCA as internal standard. All cases of cork taint observed during organoleptic assessment of 374 bottled wines sealed with cork closures could be attributed, at least in part, to the presence of TCA. There was considerable variation in the distribution of TCA and other chloroanisoles between wine and cork. In many cases, chloroanisoles were found only in the cork and had not leached into the wine. TCA was found in every cork analysed and was accompanied by varying amounts of 2,4- or 2,6-dichloroanisoles, 2,3,4,6-tetrachloroanisole and pentachloroanisole, indicating more than one origin for these compounds. Five corks were dissected and more TCA was found in the outer 2 mm of the corks than in the inner portions.

The deuterium-labelled standards, [$^2H_3$]-guaiacol, [$^2H_3$]-4-methylguaiacol, [$^2H_4$]-4-ethylphenol, [$^2H_4$]-*cis*-oak lactone, [$^2H_4$]-*trans*-oak lactone and [$^2H_3$]-vanillin, were synthesised and utilised in a new method employing gas chromatography-mass spectrometry to determine the concentration of guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *cis*- and *trans*-oak lactone and vanillin in wine or extracts of oak shavings in a single analysis. The method can employ either liquid-liquid extraction or solid-phase microextraction (SPME), and is rapid, robust, precise and accurate. Under certain conditions there was artefactual 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. These artefacts could be avoided by using SPME or by using liquid-liquid extracts with pentane or pentane:ether (2:1) injected at 200°C providing spot checks using SPME were done.

Oak volatile concentrations in wines stored in American and French new and used oak barrels were measured. In general, wine stored in new oak contained more guaiacol, 4-methylguaiacol, *trans*- and *cis*-oak lactone and vanillin and less 4-ethylphenol and 4-ethylguaiacol than did wine stored in used barrels. Wine stored in shaved and refired barrels contained up to 85% less 4-ethylphenol and 4-ethylguaiacol than wine stored in barrels of the same origin (French or American) that had not been shaved. As a separate trial, a 1998 Shiraz red wine was aged in new 300 litre oak barrels, of either French or American origin, made by 11 different cooperages. For some

cooperages, significant differences were observed in the concentrations of guaiacol, 4-methylguaiacol, *trans*-oak lactone or vanillin between fine and medium grained oak. In all cases where a difference was observed, the concentration was higher in the fine grain oak.

61 different commercial bottled single-variety Australian red wines were analysed. *Cis*-oak lactone was of primary importance to the aroma and flavour of the wines, 4-ethylphenol and 4-ethylguaiacol were moderately important, vanillin was of slight importance and the other volatiles were less likely to impart significant effects. Some varietal trends were observed. There was more guaiacol in the Shiraz wines than that of the other varieties; more 4-ethylphenol in Cabernet Sauvignon than Pinot Noir and Shiraz; less *cis*-oak lactone in Pinot Noir than Shiraz and Cabernet Sauvignon.

The effects of different heating temperatures on French and American oak in the presence of air compared to argon was investigated. Heating resulted in marked increases in the concentrations of guaiacol, 4-methylguaiacol and vanillin, with more formed at the higher temperature. About twice as much guaiacol, 4-methylguaiacol and vanillin were formed with heating in the presence of air.

The concentrations of guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone and vanillin in model wine extracts of oak shavings continued to increase over time after the oak shavings were removed. The extent of this evolution varied according to the analyte, with the largest increases observed for vanillin (up to 2.4 times the initial concentration when the shavings were removed). Air had a notable effect on the extent of the generation of these volatiles. This effect was small (< 20%), except for vanillin, in which the values could be enhanced by 10-50%.

Hydrolytic studies showed that coniferaldehyde is not a significant contributor to the formation of vanillin in wine.

Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

23/4/2000

Alan Pollnitz Date

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Publications

Refereed Journals:

1. Pollnitz, A.P., Pardon, K.H., Liacopoulos, D., Skouroumounis, G.K. and Sefton, M.A. (1996) The analysis of 2,4,6-trichloroanisole and other chloroanisoles in tainted wines and corks. *Aust. J. Grape Wine Res.,* **2**, 184-190.

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9. Pollnitz, A.P, Liacopoulos, D., Spillman, P.J., Skouroumounis, G.K., Pardon, K.H., Jones, G.P. and Sefton, M.A. (1999) The analysis of oak-derived flavour compounds in wine by stable isotope dilution. *In: Blair, R.J., Sas, A.N., Hayes, P.F., Høj, P.B., eds. Proceedings of the tenth Australian Wine Industry Technical Conference, 2-5 August 1998, Adelaide, SA: Australian Wine Industry Technical Conference Inc.,* 262.

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12. Pollnitz, A.P., Pardon, K.H. and Sefton, M.A. (2000) 4-Ethylphenol, 4-ethylguaiacol and oak lactones in Australian red wines. *Aust. Grapegrower Winemaker*, **438**, 45-52.

Chapter 1
Introduction

1.1 Wine composition and flavour

Of all alcoholic beverages, wine displays perhaps the greatest variation in flavour. Subtle nuances of colour, aroma and flavour create a unique character for each wine. Several hundred volatile components have been identified in grapes and wine and many of these are important to wine aroma and flavour (eg. Schreier 1979, Rapp and Pretorius 1989).

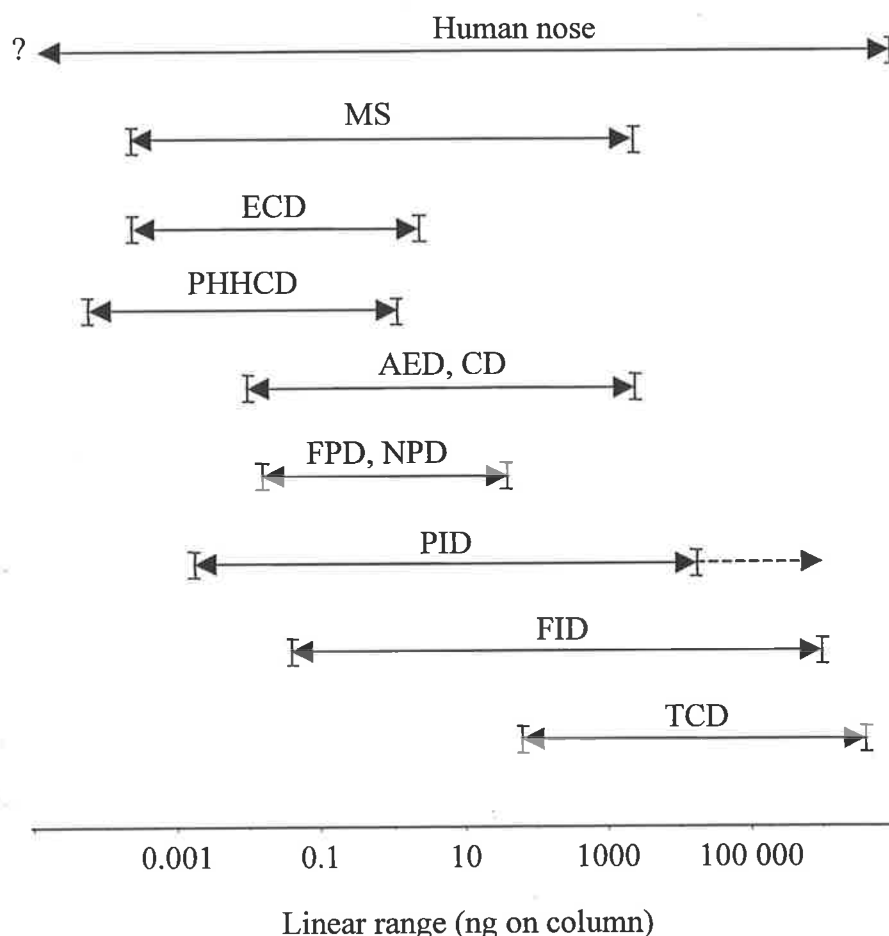
In general terms, there are several classes of compounds affecting the aroma and flavour of wine, and these come from several origins, eg. grapes, fermentation (often in contact with oak), microbiological interactions and contamination from external sources during bottling, storage or transportation. The grape-derived class of aroma and flavour compounds includes monoterpenes, shikimate metabolites, norisoprenoids, aliphatic and benzenoid compounds (eg. Sefton 1998, Sefton *et al.* 1996, Skouroumounis 1991). During fermentation, compounds are formed in wine from yeast and other micro-organisms, and the 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 flavour components. Finally, the wine is stored in bottles where more compounds are formed during maturation. Here the closure can be important as contaminants in the cork, which is provided from the bark of the cork oak species *Q. suber*, can sometimes impart unpleasant aroma and flavour volatiles into the wine. Reliable methods are needed to accurately quantify wine components in order to understand the relationship of these compounds to flavour and to investigate the effects of viticultural and oenological practices on the concentrations of these compounds.

This thesis describes the development and use of stable isotope dilution analysis (SIDA) techniques for measuring wine components associated with oak-barrel maturation (ie contact with oak products) and with taint derived from cork closures during bottle maturation.

1.2 Accurate quantification of volatile components affecting wine aroma and flavour

In general, gas chromatography (GC) is the technique of choice for the analysis of volatile compounds in wine. GC has many more theoretical plates than High Performance Liquid Chromatography (HPLC) and a much higher signal to noise ratio. GC offers a choice of injection techniques (eg split, splitless/split, pulsed splitless/split, on-column, liquid, headspace, solid phase microextraction {SPME}) and can be coupled to a wide range of detectors (as explained in further detail later in this section). A wide variety of GC columns are available, which allows tailoring of analytical methods to resolve and quantify compounds of interest, taking advantage of the analytes affinity (or lack thereof) to a bonded phase, or the boiling points of the compounds, or a combination of both. By their very nature, volatile compounds will be volatilised in the GC injector, leaving less volatile wine components (eg polyphenols, tannin, coloured pigments) behind. Experienced GC operators also take advantage of the injector block of the GC, optimising parameters (including injection temperature, speed, duration, pressure programming, solvent focussing on the column) to suit the analytical application. Wine volatiles are almost invariably analysed using a gas chromatograph (GC) coupled to a detector, commonly a flame ionization detector or mass spectrometer, but other detectors can also be used. The approximate linear ranges of these are shown in Figure 1.1. These ranges are typical for 'benchtop' detectors of the types shown. For some detectors (eg MS and AED), it is possible to gain greater sensitivity with more expensive instruments.

Figure 1.1 **Approximate linear dynamic ranges of various GC detectors**



MS = Mass Spectrometer

ECD = Electron Capture Detector

PHHCD = Polyhalogenated Hydrocarbon Chemiluminescence Detector

AED = Atomic Emission Detector

CD = Chemiluminescence Detector

FPD = Flame Photometric Detector

NPD = Nitrogen Phosphorous Detector

PID = Photoionization Detector

FID = Flame Ionization Detector

TCD = Thermal Conductivity Detector

Figure 1.1 was constructed from data obtained in Driscoll 1976, Eckert-Tilotta *et al.* 1992, Fine and Roundbehler 1975, Hutte *et al.* 1986, Yamada *et al.* 1982 and work done by the author in this laboratory.

Overall, mass spectrometry is the best detector for GC as it has a good linear range, low level of detection, and unrivalled confirmation of identity, as each compound has its own fragmentation pattern or spectral fingerprint. Schreier (1979), citing 67 references, lists 318 neutral volatile compounds identified and/or quantified in wine, of which 237 were analysed by GC (using various detectors) with 203 by GC/MS.

Accurate quantification of volatile wine components affecting aroma and flavour is a difficult task. Experience in this laboratory has indicated that many wine volatiles are susceptible to degradation reactions or loss during isolation and concentration. Many hypotheses are based on limited data only, as acquisition of the data is time consuming. There is a need for more rapid and accurate analytical methods. Even the mere quantitative determination of a major component of alcoholic beverages, ie ethanol, is subject to sources of error (Lie *et al.* 1970). There is much greater potential for error in the measurement of more minor components, such as oak-derived volatiles (at the part per billion or μg/L level) or chloroanisoles (at the part per trillion or ng/L level). A report by Rehberger *et al.* (1970) giving the results of collaborative analyses by GC at ten different laboratories, showed great variation from laboratory to laboratory, even when individual laboratories showed reasonable reproducibility (2-4% coefficient of variation) between the same method or different methods within the same laboratory (Engan 1971). Within the same laboratory, using the same operator throughout the analyses was a very important factor in obtaining consistency (Engan 1971).

Determinations are needed not just in simple solutions such as water, but in more complex matrices, such as wine. Standards used to calibrate such analyses are usually present in a matrix very different to that of the real samples - for example, most assays use a calibration mix of pure reference standards made up in a pure solvent (eg Urruty and Montury 1996, Mosedale and Ford 1996, Cerny and Grosch 1993, Miller and Stuart 1999, Aubry *et al.* 1997, Kotseridis *et al.* 1998a&b, Chatonnet and Dubourdieu 1998b), rather than spiked standard additions to real sample matrices (at a range of concentrations spaced throughout the likely range of results) extracted and analysed under exactly the same conditions as the real samples reported (eg Pollnitz *et al.* 1995, Hawthorne *et al.* 1992, Vitali *et al.* 1998, Lacassie *et al.* 1998, Kaufmann 1997, Amon

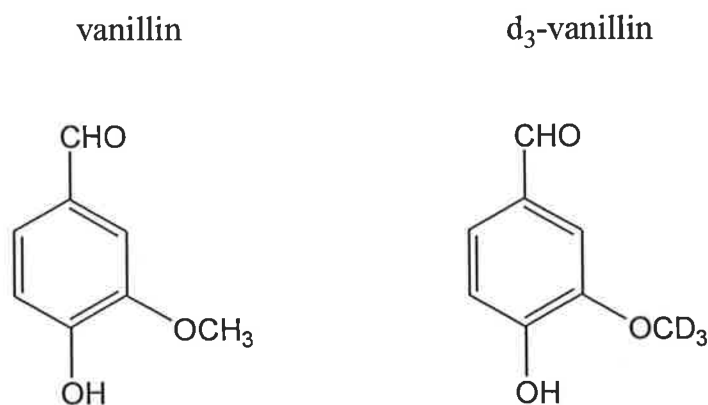
et al. 1989). In many cases, no reference is made to the matrix used for calibrants at all (eg. Swan 1997, Waterhouse and Towey 1994, Von Sydow 1975, Vas *et al.* 1998). Commonly, in the literature the standards chosen are not chemically identical, but rather just as similar as is convenient (eg. Pollnitz *et al.* 1995, Urruty and Montury 1996, Vas *et al.* 1998, Kaufmann 1997, Lacassie *et al.* 1998, Kotseridis *et al.* 1998a, Waterhouse and Towey 1994, Towey and Waterhouse 1996a, Guichard *et al.* 1995, Feuillat *et al.* 1997, Simpson *et al.* 1986, Rocha *et al.* 1996, Peréz-Coello *et al.* 1999, Gelsomini *et al.* 1990, Chatonnet and Dubourdieu 1998b, Amon *et al.* 1989). Often only one standard is used to represent a wide range of compounds analysed in a multi component assay (eg Pollnitz *et al.* 1995, Towey and Waterhouse 1996a, Lacassie *et al.* 1998, Urruty and Montury 1996, Vas *et al.* 1998, Kaufmann 1997, Rocha *et al.* 1996, Chatonnet and Dubourdieu 1998b). Frequently a standard is added only after extraction is completed and thus only serves to aid accuracy in the instrumental analysis but gives no aid whatsoever in determining the accuracy of sample extraction and purification (eg. Guichard *et al.* 1995, Feuillat *et al.* 1997). At times, there is no mention of a standard used at all (eg. Vitali *et al.* 1998, Swan 1997). Often no repeatability data is given (eg. Pollnitz *et al.* 1995, Semmelroch *et al.* 1995, Sen *et al.* 1991, Waterhouse and Towey 1994, Swan 1997, Guichard *et al.* 1995, Von Sydow 1975, Cerny and Grosch 1993, Rocha *et al.* 1996, Peréz-Coello *et al.* 1999). Engan (1971) states that occasionally misleading correlation coefficients and / or standard deviations are presented that seem to have no connection with the chromatograms simultaneously presented. Occasionally good extraction technique is not coupled with the best instrumental technique. For example, the operating conditions reported by Semmelroch *et al.* (1995) and Blank *et al.* (1992) would only result in five data points per peak in each extracted ion chromatogram, and thus induce potential for error in reproducible integrations from run to run (unsurprisingly no repeatability data was given). As another example, excessive column bleed and thus negative effects on recoveries, repeatabilities and level of detection, particularly on later-eluting compounds, is evident in Lacassie *et al.* (1998.) In addition, often, insufficient data is shown to make a judgement on the validity of instrumental techniques (eg Waterhouse and Towey, 1994; Swan, 1997).

It would obviously be an advantage if all the different methods currently in use could be reduced to a few recommended robust methods that would give good accuracy and reproducibility, even when used in different laboratories.

1.2.1 Stable isotope dilution analysis (SIDA)

Stable isotope dilution analyses (SIDA) using isotopically–labelled internal standards are the most accurate methods for the quantification of aroma and flavour components in foodstuffs, and are finding increasing use (eg Semmelroch *et al.* 1995, Guth and Grosch 1990, Masanetz *et al.* 1995, Schieberle and Grosch 1987, Sen *et al.* 1991, Sen and Grosch 1991, Hawthorne *et al.* 1992.) The procedure (SIDA) employs the addition of a precisely measured amount of an isotopically labelled standard to a precisely measured amount of the matrix of interest (wine in the case of this thesis). The technique has been used for over a decade to determine the concentration of methoxypyrazines in grapes and in wine at the ng/L (ppt) level (Harris *et al.* 1987, Allen and Lacey 1993 & 1998, Allen *et al.* 1994, 1995a&b, Kotseridis *et al.* 1998b). In addition to the work described in this thesis, recently SIDA techniques have been described for the quantitation of ethyl dihydrocinnamate, ethyl cinnamate, methyl anthranilate and ethyl anthranilate (Aubry *et al.* 1997), β-damascenone, α-ionone and β-ionone (Kotseridis *et al.* 1998b), and diacetyl (Hayasaka and Bartowsky 1999) in wine.

As an example of SIDA, Semmelroch *et al.* (1995) used the isotopically labelled compound d_3-vanillin to measure vanillin in coffee.



Vanillin and d_3-vanillin have virtually identical physical and chemical properties. They have the same solubility, melting points, boiling points and retention times in gas chromatography (to within 0.02 min, with d_3-vanillin having the lower retention time if any difference is visible). The only way to tell the two compounds apart is by a suitable instrumental technique, eg ^{1}H nmr where the singlet due to the three methoxy hydrogens will be absent from the d_3-vanillin spectrum, or by mass spectrometry. For positive ion electron impact / mass spectrometry (EI/MS) the molecular ions are *m/z* 155 for d_3-vanillin and *m/z* 152 for vanillin. The M-1 cleavage for the loss of the aldehydic hydrogen gives m/z 154 for d_3-vanillin and *m/z* 151 for vanillin. Some of the other fragmentations are not useful for discriminating between the two compounds because once the methoxy group is lost, the isotopic labels are lost with it and the ions observed are identical for both compounds. Mass spectrometry has the added advantage of being able to determine the relative amounts of each compound present in a mixture of vanillin and d_3-vanillin.

d_3-Vanillin is the internal standard used in the stable isotope dilution analysis (SIDA) of vanillin in wines, described in this thesis.

Semmelroch *et al.* (1995) analysed odourants in coffee by both stable isotope dilution assays and conventional methods. Many of the results differed by factors of up to 50%. In another comparative experiment, Guth and Grosch (1994) used the same methodology to quantitate caffeic and ferulic acid in oatmeal utilising both isotopically labelled standards and a non-isotopically labelled standard. The relative recovery (using the non-isotopically labelled standard) was a mean of 2% compared to a mean of 95% (with the isotopically labelled standards). Accuracy was similarly compromised when the non-isotopically labelled standard was used.

The advantage of SIDA is that the internal standard is virtually identical chemically to the substrate being assayed, and therefore the accuracy of the analysis is not reduced by inefficiency in isolation or by analyte decomposition. The theory holds that no matter what happens during sample preparation and analysis, the ratio of the isotopically labelled standard to its non–isotopically labelled analogue remains the same. This includes extraction, concentration and analysis. An advantage of this is that complete

extraction of the analyte of interest from the matrix is no longer a necessity. Methods can be streamlined so that extractions can be done more quickly and with less sample. For example the traditional vanillin methods employed by Sefton *et al.* (1993a&b) used exhaustive continuous liquid-liquid extraction of at least 500 mL of wine with three successive aliquots of Freon F11 over 72 h, and then a concentration step was required prior to analysis by GC/MS. In contrast, the SIDA method for vanillin developed later in this thesis uses 5 mL of wine, 2 mL of ether (or pentane/ether), extraction takes a few minutes and there is no concentration step required prior to analysis by GC/MS.

1.2.2 Analysis of the headspace above wine

Gas chromatographic analysis of the headspace above wine is particularly inviting because of its simplicity and its ability to give results in which artefacts resulting from extraction and concentration are kept to a minimum (eg Bertuccioli and Montedoro 1974, Bertuccioli and Viani 1976, Teranishi *et al.* 1971, Engan 1971, Guth and Grosch 1993). This is a particular advantage when analysing volatile aroma compounds in a complex matrix, such as wine, because the most volatile compounds, including those of interest, can be separated from those of low volatility. This can theoretically increase the signal to noise ratio in instrumental analyses. Unfortunately conventional headspace concentration (eg Wang *et al.* 1983, Bertuccioli and Montedoro 1974, Bertuccioli and Viani 1976) is time consuming and can present all sorts of problems, especially with repeatability. Inside–out chromatography or SPME (Solid Phase Micro Extraction) is much quicker than conventional headspace and can be automated. SPME was developed by J. Pawlisyn in 1989 and marketed by Supelco (Bellefonte, USA) in 1993 opening the door for research applications, especially when combined with gas chromatography (eg Hawthorne *et al.* 1992, Penton 1996, Mitchell and Tame 1996 and De la Calle García *et al.* 1996.) The SPME device made by Supelco consists of a *ca* 10 mm long tube of fused silica with an adsorbent material matrix (eg. polydimethylsiloxane or carbowax / divinylbenzene) bonded to the outside. This fused silica is attached to the end of a metal fibre such that the entire assembly can be retracted into a hollow metal needle of slightly larger diameter than a standard GC

syringe. The headspace of the sample is sampled by puncturing the sample vial with the metal needle, extending the fibre into the headspace above the vial and waiting for the analytes in the gas phase above the sample to concentrate on the fibre. Once this is done (usually after 10-30 minutes) the fibre is retracted and the assembly functions like a GC syringe with the metal needle puncturing the septum of the GC injector port, followed by extension of the fibre in the injector and the subsequent thermal desorption onto the GC column. SPME also has the advantage of concentrating the headspace volatiles onto the fibre *in situ*, unlike more time consuming liquid / liquid extraction procedures (eg. Cutzach *et al.* 1999, Atienza *et al.* 1998, Chatonnet and Dubourdieu 1998a&b, Spillman *et al.* 1998b, Sefton *et al.* 1993a&b, Simpson *et al.* 1986), solid phase extraction or traditional static or purge and trap headspace (eg. Bertuccioli and Montedoro 1974, Bertuccioli and Viani 1976, Engan 1971, Conner *et al.* 1998, Wang *et al.* 1983) where concentration steps are often required prior to sample analysis. Unlike static headspace sampling where volatility of the components is the major factor, the SPME fibre is more selective, ie the nature of the bonded phase and the time of extraction affect the absolute and relative concentrations of the volatiles. This is both an advantage and a disadvantage. On the positive side, SPME can be more selective than static headspace, in that fibres and extraction conditions can be manipulated to favour the analytes of interest over other volatiles present in the matrix. The disadvantage is that a less true picture of the relative concentrations of different volatiles to each other may be observed, unless the analyst uses reliable internal standards and / or thoroughly calibrates the method over a variety of conditions. Successful headspace analysis, whether by SPME or conventional methods, relies on an effective headspace injector design on the GC and reproducible agitation of the sample, temperature of the sample and headspace and the time of extraction. Ionic strength and the levels of polymeric material are always variable in wines and have considerable effect on the headspace concentration of volatiles. It is not easy to guarantee reproducible sample matrix, agitation, temperature and time control during headspace extraction, even with automated extractors, but the use of isotopically labelled standards solves this problem as the ratio of the analyte to its isotopically labelled internal standard will remain constant regardless of the extent or rate of headspace extraction.

1.2.3 GC analysis using an olfactory (sniff) detector

A valuable analytical tool for detecting and identifying new aroma compounds is GC/sniff (eg. Amon *et al.* 1989, Vitzthum 1976, Milo and Grosch 1993, Holscher *et al.* 1992, Guth and Grosch 1993), ie. the coupling of gas chromatography (GC) with the human nose as a detector. GC/sniff is also known as GC/Olfactory Detection. Basically, the effluent from the GC column is split into two columns at the detector end by the use of a Y–shaped zero dead volume connector. One end of the column goes to a conventional detector, such as a flame ionisation detector, flame photometric detector or mass spectrometer, the other end of the column goes to a heated sniffing cup, through which a stream of humidified air is passed. Extracts should be run more than once and assessed by more than one sniffer because aroma thresholds and descriptors vary from person to person. This practice also shows which aroma components are more important (more than one assessor detects it) and which are less important (only one assessor detects it).

The main limitation of GC/sniff is that what comes out of the heated sniffing cup is not limited to what is smelt normally. A feature of GC/sniff, commonly ignored by its proponents, is that many compounds are not very volatile in the matrices in which they occur (eg wine), but are typically ~90-100% vaporised in the injector block of the GC. For example, cyclotene and maltol have burnt woody and toasty aroma descriptors via GC/sniff and it has been suggested that these two compounds are responsible for sweet, burnt woody and toasted wood aromas in alcoholic beverages (Nishimura *et al.* 1983). However unpublished results from this laboratory show extremely high aroma detection thresholds for cyclotene (*ca* 4000 ppb) and maltol (*ca* 6000 ppb) in wine.

More recently, Chatonnet (1998) reports perception thresholds in 12% aqueous alcohol of *ca* 2000 ppb for cyclotene and *ca* 5000 ppb for maltol. This data indicates cyclotene and maltol are not at all significant to wine aroma, as their concentrations present in wine are far below these thresholds. These two compounds are highly water soluble and presumably have low partial vapour pressures in wine. As another example, ethyl dihydrocinnamate, ethyl cinnamate, methyl anthranilate and ethyl anthranilate have been assumed to be important in the aroma of Pinot Noir wines, based on GC/sniff assessments, but are actually present at levels below half their sensory thresholds in water (Aubry *et al.* 1997).

In some cases, a characteristic aroma can not be found via GC/sniff in any one part of the gas chromatogram but the familiar characteristic aroma results from the concerted effects of many compounds (Vitzthum 1976). Several studies (eg. Lawless 1986, Meilgaard 1989, Rothe 1988, Wang *et al.* 1983, Laing 1987) report combinatory effects between two or more volatile components which can enhance or reduce the overall aroma effect.

GC/sniff can nevertheless be useful for highlighting some compounds for further investigation.

1.3 Taint compounds derived from cork closures and volatile aroma compounds derived from maturation in oak barrels

Oak is the common name used for the trees of the several hundred identified species of the genus *Quercus* and their vast number of hybrids (eg. Schahinger 1991, Maga 1989b, Howes 1953). Oak species can be broadly divided into two types, red oak and white oak. Although the timbers are difficult to distinguish from each other by a physical inspection, the important commercial difference between them is that red oak is porous but most white oaks are not. The most well known red oak is *Quercus suber.* Traditional wine corks are produced from the bark of this species.
White oak is the term used to describe the products of less than twenty species of the genus *Quercus.* In the United States of America, the dominant species is *Q. alba.* Throughout Europe, the dominant species are *Q. petraea (Q. pedunculate)* and *Q. robur (Q. sessiliflora, Q. sessilis)*, the brown or English oak. (Singleton 1973, Aiken and Noble 1984a, Puech and Moutounet 1990, Schahinger 1991, Waterhouse and Towey 1994). Other woods are less suitable for tight cooperage, having either less desirable mechanical properties or imparting unpleasant aromas into wine; these can only be used to store wine for very short periods, or need to be coated with a paraffin–like wax (eg Singleton 1973, Schahinger 1989).

1.3.1 Corks and 'cork taint'

The main interest in the volatiles derived from cork is that they can sometimes impart an unwanted flavour or 'taint'. Such taint compounds are presumed to be formed microbiologically either from man-made chlorophenolic precursors or natural metabolites.

The properties of cork as a sealant have been recorded for millennia. Cylinders cut from the bark of the cork oak, *Quercus suber,* have been used as closures for wine since about the year 1700, when the regular packaging of wine in glass bottles commenced (Lee *et al.* 1984, Sharf and Lyon 1958, Grossmann *et al.* 1996).

Corks are still widely regarded as an inert closure that acts only as a seal against wine leakage and oxidation. A good cork should have the following attributes: impermeability to air and liquids (preventing wine oxidation and loss of desirable aroma volatiles), the ability to adhere to the glass surface of the bottle neck, compressibility, resilience, the correct size and shape and chemical inertness (see Amon and Simpson 1986, Rocha *et al.* 1996).

Sometimes the quality of a bottled wine is lessened due to a contamination of the cork. The troubles caused by "cork taint" or "cork off–aroma" are believed to have existed since corks were first used at the end of the 17th century. Prevention methods had already been published in 1904 (Bordas). The problem of cork taint is regarded as among the most serious confronting the wine industry today (eg. Simpson 1990, Shaw 1995, Baldwin 1993, Smith 1992). Estimates of the incidence of cork taint range from 2–10% of all bottled wine. Although cork taint is unacceptable to winemakers, consumers will often tolerate such spoilage of 2–10% of all bottles of wine because they do not recognise it for what it is. It is unlikely that the same consumers would tolerate their cars, televisions or refrigerators not working 2–10% of the time! There are metal seals superior to cork. Previous attempts to use closures other than cork, however, have been unsuccessful from a marketing point of view. Consumers associate a bottled wine 'without a proper cork' as a low quality wine (see Lee *et al.* 1984, Joubert 1981). A

concern of winemakers is that even where cork taint is too weak in intensity to be identified as such, it can nevertheless mask positive aromas and make a very good wine smell like a poor quality wine (Simpson 1990, Baldwin 1993). Consumer and industry education is one possible way of solving the problem of cork taint, albeit a frustrating and time consuming solution. It may be more viable to isolate, identify and understand the causes of cork taint. Opinion is divided over what causes cork taint and many opinions are biased by location of the opinionist, ie. cork growers blame the cork manufacturers and winemakers (Leske 1996); winemakers blame the other two.

1.3.2 The causes of cork taint

2,4,6–Trichloroanisole (TCA) is almost universally regarded as the prime cause of cork taint (eg. Amon *et al.* 1989, Duncan 1995). It was first cited as a source of cork taint by Buser *et al.* (1982). There is some evidence that other cork–derived compounds, both identified (eg. Amon *et al.* 1989) and unidentified (Duncan 1995) may also induce dusty/musty/baggy/earthy taints in wine. Nevertheless, TCA is regarded as the prime cause of cork taint because of its extremely low odour threshold in wine of 1.4 to 4.6 ng/L (Duerr 1985, Liacopoulos *et al.* 1999) and its high incidence in wines and corks.

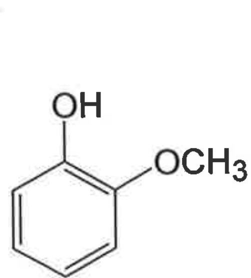
Some of the compounds thought to contribute to "cork taint", and their corresponding aroma thresholds are shown in table 1.1. Their structures are given in the subsequent figures (1.2 and 1.3).

Table 1.1

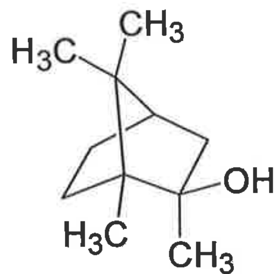
Potential contributors to cork taint and their odour detection thresholds

Compound	Aroma Descriptors	Threshold	Medium	Ref.
guaiacol	smoky	20 000 ng/L	white wine	1
2-methylisoborneol	earthy/musty	30 ng/L	white wine	2
geosmin	earthy/musty	25 ng/L	white wine	2
1–octen–3–one	mushroom	20 ng/L	white wine	2
1–octen–3–ol	mushroom	20 000 ng/L	white wine	2
2,4–dichloroanisole	all of these	400 ng/L	water	3
2,6–dichloroanisole	smell	40 ng/L	water	3
2,4,6–trichloroanisole	dank,	**1.4 ng/L**	**white and**	**4, 5**
	musty	**to 4.6 ng/L**	**red wines**	
2,3,4,6–tetrachloroanisole	and	25 ng/L	white wine	2
pentachloroanisole	dusty	4000 ng/L	water	6

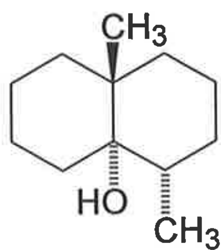
{Table compiled from references; Simpson *et al.* 1986 (1), Amon *et al.* 1989 (2), Griffiths 1974 (3), Duerr 1985 (4), Liacopoulos *et al.* 1999 (5), Curtis *et al.* 1974b (6).}



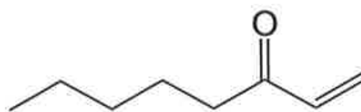
guaiacol



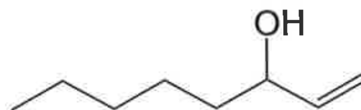
2-methylisoborneol



geosmin

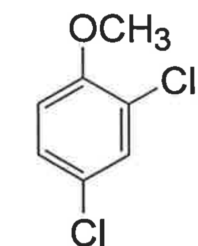


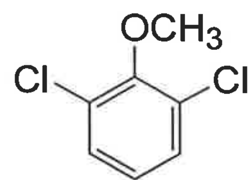
1-octen-3-one

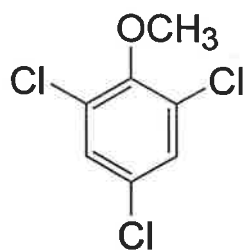


1-octen-3-ol

Figure 1.2 Structures of some compounds thought to contribute to cork taint (excluding chloroanisoles)







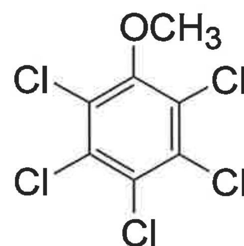


Figure 1.3 Structures of the five common chloroanisoles

DCA = dichloroanisole

TCA = trichloroanisole

TeCA = tetrachloroanisole

PCA = pentachloroanisole

1.3.3 The origins of trichloroanisole and other chloroanisoles

Several authors (eg Lee *et al.* 1984, Simpson 1990, Sponholz and Muno 1994) have reviewed the processes thought to contribute to the accumulation of TCA and other chloroanisoles in corks. All proposed pathways to chloroanisoles include microbiological methylation of chlorophenols. This transformation is fundamentally a means by which the microorganisms convert chlorophenols into a less lethal metabolite and thus detoxify their environment (Whitfield *et al.* 1991a and 1991b, Tindale *et al.* 1989).

The chlorophenolic precursors to TCA in corks are probably derived from more than one source. The direct precursor to TCA is 2,4,6–trichlorophenol (TCP). Buser *et al* (1982) assumed that TCA in corks was a result of chlorine bleaching in the processing of cork. Sponholz and Muno (1994) showed that chlorine–bleached corks contained significantly higher levels of TCP than unbleached controls, for the corks they analysed.

Polychlorophenol biocides such as pentachlorophenol (PCP) are another possible source of chlorophenolic precursors to chloroanisoles in corks. These biocides are used as a wood preservative in cork oak forests and on the wooden floors of shipping containers (Simpson 1990). Maarse *et al.* (1985) blamed the use of chlorophenolic biocides in the forest for being the prime cause of cork taint. Industrial preparations of PCP, the most commonly used chlorophenol biocide, are contaminated with small amounts of 2,3,4,6–tetrachlorophenol (TeCP) and 2,4,6–trichlorophenol. Other commercially used wood preservatives also have TCP present (Simpson 1990) or derivatives of TCP which can be degraded by microorganisms to TCP (Lee *et al.* 1984) and thus to TCA.

It has also been proposed that highly chlorinated biocides (eg PCP and lindane (γ-tetrachlorocyclohexane) can also be dechlorinated and thus converted to TCA by microorganisms (Sponholz and Muno 1994, Maarse *et al.* 1988). Neidleman and Geigert (1986) investigated some mechanisms of microbiological dehalogenation.

Engst *et al.* (1975) demonstrated that pentachlorophenol (PCP), 2,3,4,6-tetrachlorophenol (TeCP) and 2,4,6-trichlorophenol (TCP) were made from pentachlorobenzene by a lindane–reducing fungal culture. Other meta-chlorinated trichlorophenols were also produced by similar mechanisms. It is not clear, however if TCP and TeCP were obtained from PCP or from the oxidation of the other observed degradation products 1,3,5-trichlorobenzene and 1,2,3,5-tetrachlorobenzene.

Maarse *et al.* (1985) analysed the chloroanisoles in jute sacks that were the subject of a ten year investigation into their off–odour. Meta–chlorinated chloroanisoles were generally observed, plus greater quantities of TCA, tetrachloroanisole (TeCA) and pentachloroanisole (PCA). Again, it is not clear if all of these products came from the same degradation pathway, or if the meta–chlorinated chloroanisoles came from dechlorination of PCP followed by subsequent methylation, while the TCA and TeCA were made only from the methylation of the TCP and TeCP contaminants of commercial PCP.

Ide *et al.* (1972) studied the decomposition of PCP by soil samples. Interestingly, TeCP but not TCP was detected amongst the degradation products. The other degradation products were all chlorophenols chlorinated in one or both meta– positions. This tendency to retain chlorines meta– to the phenolic substituent during reductive dechlorination of chlorophenols has also been observed by several other authors (eg. Mikesell and Boyd 1985 and 1986, Boyd and Shelton 1984 and Weiss *et al.* 1982).

Other haloanisoles, such as bromoanisoles also have musty / dusty aromas and may also contribute to cork taint, although this is apparently a much rarer phenomenon. Little research has been published in this area (Whitfield *et al.* 1997).

Rocha *et al.* (1996) found that autoclaving reduced the amount of musty, mouldy or related odour compounds present in the cork. However, more than one autoclaving procedure was not recommended even though their second autoclaving process removed an additional 71% of the total amount of musty / mouldy compounds. Rocha *et al.* (1996) stated that significant amounts of furans, 1–octanol and lignin related

compounds of benzaldehyde and benzyl alcohol were generated and that the generated compounds were regarded as providing unacceptable sensory taints in their own right (albeit without any supporting evidence). Their formation was attributed to further degradation of the lignin and other cork polymers present by the more intense treatment.

Methods of analysis for TCA and other chloroanisoles are discussed in Chapter 3.

1.3.4 Flavours imparted to wine by maturation in oak barrels

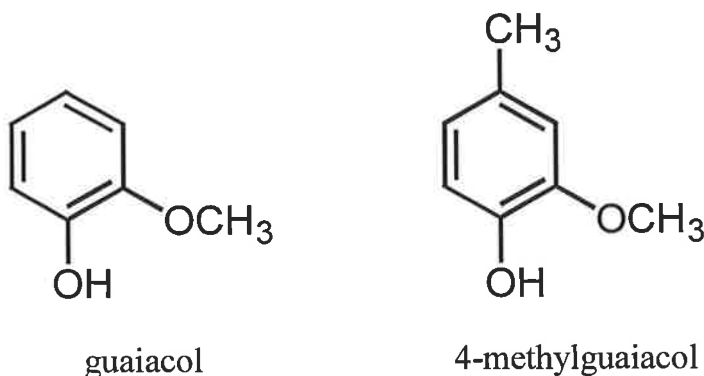
The following section focuses on the nine compounds derived primarily during the barrel maturation of wine, for which new analytical methods were developed during the course of this work. Several other oak compounds, which were not the subject of the analytical investigations within this thesis, are also described briefly. Specific methods of analysis for these volatiles are discussed in Chapter 4.2.4.

Although barrels were originally regarded as little more than inert containers they are now known to enhance wine flavour. Wine is traditionally stored in oak barrels or casks during a period of maturation, lasting from a few weeks to a few years. The purpose of this ageing in wood is to improve and enhance the sensory quality of the wines, modifying the aroma and making it more complex and pleasant (eg. Chatonnet *et al.* 1991, Maga 1989b, Guymon and Crowell 1968).

The oak composition and condition in which wine maturation takes place is of primary importance (Mosedale and Ford 1996, Williams 1983). Several aroma compounds identified in wine are derived principally during the process of oak barrel maturation of the wines. Some of these compounds are listed in the following sections. Some oak–derived flavour compounds are formed from thermal degradation of lignin or cellulose and hemicellulose (eg Towey and Waterhouse 1996b, Wittowski *et al.* 1992, Puech 1981, Maga 1989b, Nishimura *et al.* 1983, Reazin 1981) and extracted during wine storage. The charring or toasting of oak barrels transforms some of the lignin into forms that react more readily with wine than does unheated lignin.

Hundreds of oak compounds have been identified in the literature (eg. Maga 1989b, Chatonnet and Boidron 1989a&b, Chatonnet 1991, Chatonnet *et al.* 1991, 1992a, 1995, Chatonnet and Dubourdieu 1998a&b, Cutzach *et al.* 1997, 1999). However only a few are considered to have a significant effect on wine quality and flavour. The relative concentrations of these important compounds, and the effect on wine quality and flavour, due to variations in oak origin, seasoning, coopering, microbial interaction (such as in malolactic fermentation) and other industry practices have been studied by many authors (eg Chatonnet and Dubourdieu 1998a, Chatonnet *et al.* 1991, Boidron *et al.* 1988, Maga 1989a&b, Towey and Waterhouse 1996a&b, Howes 1953, Guymon and Crowell 1968, Sefton *et al.* 1993a&b and Spillman *et al.* 1996).

1.3.4.1 Guaiacol and 4–methylguaiacol



guaiacol 4-methylguaiacol

Guaiacol (2-methoxyphenol) has a smoky, phenolic, aromatic, burnt, burnt bacon aroma (Boidron *et al.* 1988, Blank *et al.* 1992, Holscher *et al.* 1990, Tressl 1989). The aroma threshold of guaiacol in white wine is 20 µg/L (Simpson *et al.* 1986). Guaiacol can be perceived as a smoky aroma above 95 µg/L in white wine and 75 µg/L in red wine (Boidron *et al.* 1988). 4-Methylguaiacol can be perceived as a smoky aroma above 65 µg/L in both red and white wines (Boidron *et al.* 1988). In hundreds of analyses done at this laboratory it has been observed that typically, wine matured in oak contains between 10 and 200 µg/L of guaiacol and between 1 and 20 µg/L of 4-methylguaiacol, although much higher values have occasionally been observed. Both guaiacol and 4-methylguaiacol are formed by the pyrolysis of lignin (eg Wittowski *et al.* 1992)

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. (Chatonnet and Boidron 1988, Chatonnet *et al.* 1991 and 1992a). There is some evidence that 4–methylguaiacol might be produced from vanillin by bacteria during alcoholic fermentation (Steinke and Paulson 1964). Braus and Miller (1958) speculated that phenols, including 4-methylguaiacol, might originate from the degradation of lignin by bacteria or fungi during fermentation.

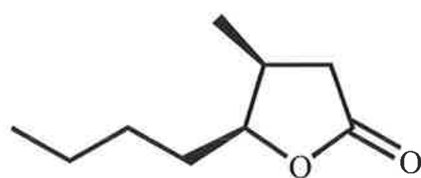
1.3.4.2 Oak lactone

The *cis*- and *trans*-isomers of 5-butyl-4-methyl-4,5-dihydro-2(3H)-furanone, ie. *cis* and *trans* β–methyl–γ–octalactone, commonly called oak-lactone or whisky lactone, are found in spirits or wines that have been in contact with oak. *Cis*- and *trans*-oak lactone were first identified by Suomalainen and Nykänen (1970a&b) in whisky and various other distilled alcoholic beverages that had been aged in oak. Kahn *et al.* (1969) were the first to report the existence of the oak lactones and their strong odour characteristics but could only tentatively assign their structure (incorrectly) as a branched 5–δ–nonalactone.

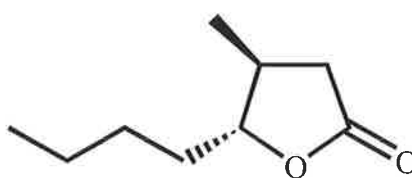
In a recent sensory study (Spillman 1998), the concentration of the *cis*-isomer of oak lactone was positively correlated with the aroma intensity of the 'coconut' descriptor in a Chardonnay wine, and with the aroma intensity of 'coconut', 'vanilla', 'berry' and 'dark chocolate' descriptors in a Cabernet Sauvignon wine. The sensory panel also preferred wines with higher concentrations of *cis*-oak lactone. Chatonnet *et al.* (1990) found that additions of a 1:1 mixture of *cis*- and *trans*-isomers (as a racemate) to a red wine enhanced 'woody', 'coconut', and 'varnish' characters, but resulted in a decrease in 'preference' at a concentration above 235 μg/L. Oak lactones have also been directly linked to the quality of oak-aged spirits (Otsuka *et al.* 1974, Reazin 1981).

Salo *et al.* (1972) quoted the odour threshold of a synthetic racemic mixture of all four isomers of β–methyl–γ–octalactone as 51 μg/L. It had by far the lowest odour threshold level out of the 24 compounds and 3 mixtures in the ester fraction they studied. Masuda and Nishimura (1971) established that the oak lactone isomers in alcoholic beverages are derived only from oak wood and this has been confirmed by Otsuka *et al.* (1974).

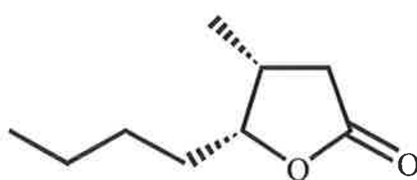
As oak lactone has no plane of symmetry and two chiral centres, it has four stereoisomers, ie. two enantiomeric pairs of diastereomers. Only the *cis* (3S,4S) and *trans* (3S,4R) isomers occur naturally and have been found in wood extracts (eg. Guichard *et al.* 1995, Ebata *et al.* 1993), while 3R diastereomers do not occur in nature (Gunther and Mosandl 1986 and 1987, Masuda and Nishimura 1971 and 1981, Guichard *et al.* 1995).



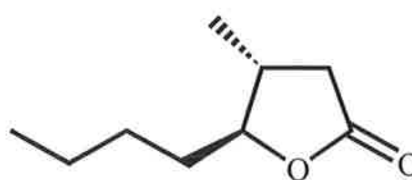
cis(3S,4S)-oak lactone



trans(3S,4R)-oak lactone



cis(3R,4R)-oak lactone



trans(3R,4S)-oak lactone

These four isomers have been converted to Mosher esters and separated by liquid chromatography (Günther and Mosandl 1987). König *et al.* (1988) obtained direct chiral separation by gas chromatography on a modified cyclodextrin phase. Chiral GC columns could be very useful in determining the enantiomeric ratios in different synthetic extracts.

There is some contradiction and confusion in the earlier literature with some authors (eg Otsuka *et al.* 1974, Salo *et al.* 1972, Masuda and Nishimura 1971) mis-assigning the *cis* and *trans* isomers of the oak lactone, calling the *cis* the *trans* and vice versa. In 1981, Masuda and Nishimura (1981) correctly assigned the isomers. They are readily distinguished chromatographically in that the *trans* isomer is eluted before the *cis* during gas chromatography and on normal phase liquid chromatographic phases. Any mis-assigned isomers in the references cited have been correctly reassigned throughout this thesis.

Guichard *et al.* (1995) observed that in commercial mixtures of *cis* and *trans* oak lactones, mixtures with a higher proportion of the *cis* oak lactones had more coconut aroma and those with a higher proportion of the *trans* oak lactones smelt more woody and herbaceous. These results are in agreement with the odour descriptions given by Günther and Mosandl (1986) for each of the four stereoisomers, separated via their Mosher esters. Anonymous (1995) gave aroma descriptors for each of the four stereoisomers individually and for the pair of *cis* isomers and the pair of *trans* isomers. Table 1.2 (following directly) is a combination of the descriptions from Anonymous (1995), Guichard *et al.* (1995) and Günther and Mosandl (1986). Thresholds for mixtures of isomers are given in table 1.3.

Table 1.2 Sensory aroma descriptors of oak lactone stereoisomers

Isomer	**Aroma descriptors**
both *trans* isomers	weedy, hay, celery, spicy, jasmine, herbaceous
both *cis* isomers	Sweet, cinnamon, jasmine, fatty, coumarin, coconut
trans-(3S, 4R)*	coconut, weedy, woody, spicy, sweet, hay, celery, jasmine
trans-(3R, 4S)	sweet, fruity, floral, hay, peach, jasmine, coconut, celery, weedy
cis-(3R, 4R)	sweet, soft, hay, coumarin, coconut
cis-(3S, 4S)*	sweet, cinnamon, soft, fatty, coconut, jasmine

* Naturally occurring stereoisomer

Table 1.3 Oak lactone aroma thresholds

Isomers	Threshold (μg/L)	Medium	Reference(s)
all four	51	34% ethanol / water	Salo *et al.* 1972
both *trans*	790	30% ethanol / water	Otsuka *et al.* 1974
both *cis*	67	30% ethanol / water	Otsuka *et al.* 1974
all four	20	water	Boidron *et al.* 1988
all four	15	model wine*	Boidron *et al.* 1988
all four	120	white wine	Boidron *et al.* 1988, Chatonnet *et al.* 1990
all four	125	red wine	Boidron *et al.* 1988, Chatonnet *et al.* 1990
both *trans*	460	white wine	Chatonnet 1991
both *cis*	67	white wine	Chatonnet 1991
all four	75	12% ethanol / water	Piggott *et al.* 1995
all four	241	white wine	Piggott *et al.* 1995
all four	853	red wine	Piggott *et al.* 1995

*model wine was 12% ethanol / water, plus 5 g/L tartaric acid, adjusted to pH 3.5 with sodium hydroxide, plus 30 mg/L SO_2

The sensory threshold data are only of limited worth: all the values shown above (Table 1.3) are from a synthetic racemic mixture of all four stereoisomers, except for Otsuka *et al.* 1974 and Chatonnet 1991 where the data is from two racemic mixtures, one of the two *trans* isomers and one of the two *cis*. In the publications where all four isomers have been separated, no threshold determinations were done. Günther and Mosandl (1986) had the skill and determination to separate and purify all four isomers but then they only did a quick study on the aroma of the individual isomers at a concentration of 10 000 ppb! A thorough sensory threshold study on the individual isomers has yet to be undertaken, but would be a worthwhile exercise.

The level of *trans*-oak lactone typically found in barrel matured wines varies in concentration from less than 20 ppb up to over 400 ppb. The level of *cis*-oak lactone typically found in barrel matured wines varies in concentration from less than 20 ppb up to over 1000 ppb. Otsuka *et al.* (1974) found that at their threshold concentrations, the aroma of the *trans* oak lactone (at 790 ppb) was preferred over the *cis* (at 67 ppb) by 22 of their 24 judges (after reassigning the isomers correctly).

Maga (1989a & b) postulated that the oak lactone isomers were formed through lipid oxidation. He also showed that 'charring' the oak wood roughly tripled the amount of oak lactone present, although nowhere does he explain what 'charring' was, or to what extent the oak wood was heated or charred. He also stated that sapwood had less oak lactone than heartwood and that oak lactone extraction during ageing in barrels was linear with time.

Otsuka *et al.* (1974) found that the amounts of free and bound *cis*–oak–lactone present in the wood varied over a wide range (from 0 to 27.3 mg *cis*–oak lactone per 100 g wood) according to the origin and species of the *Quercus* wood. 'Bound' oak lactone refers to the amount of extra oak lactone liberated from the powdered oak wood extract after strong acidic reflux. This extra oak lactone is related to the amount of a hypothetical oak lactone "precursor" present in the oak wood (Otsuka *et al.* 1974). Otsuka *et al.* (1974) stated that the quality of barrel wood could be directly related to the

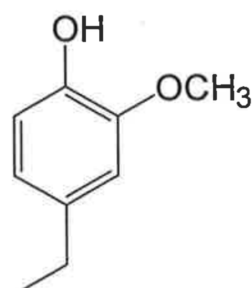
amount of oak lactone and its "precursor". Although the bound form of oak lactone is commonly referred to as a "precursor" in the literature (eg. Chatonnet 1998) the formation of oak lactone from this bound form during the growth of oak trees, wood drying, barrel maturation, vinification or wine conservation (bottle ageing) has never been demonstrated.

Otsuka *et al.* (1980) and Reazin (1981) allude to a possible relationship between oak lactone, tannins and ethyl lignin formation. Otsuka *et al.* (1974) also concluded that French oak had less oak lactone than American oak for the samples they analysed, which is in agreement with Guymon and Crowell (1972) and Towey and Waterhouse (1996a&b). However the influence of coopering heat and seasoning may affect the levels of oak lactone found as much as does the origin of the wood, and that variation within a region can be as great or greater than that between regions (Spillman *et al.* 1996, Sefton *et al.* 1993a & b).

The measurement of the oak lactones in wines and wood lots is likely to be important to quality determination and to understanding the sensory characteristics of wine.

1.3.4.3 4–Ethylphenol and 4–ethylguaiacol

4-ethylphenol



4-ethylguaiacol

The yeast *Brettanomyces / Dekkera* has been described as "one of the most complex and controversial issues encountered in the making of red wine" (Olsen 1994). Among the aroma compounds produced in red wines from these yeasts are the volatile phenols, 4–ethylphenol and 4–ethylguaiacol (eg. Chatonnet *et al.* 1992b and 1995, Towey and

Waterhouse 1996b), which are formed from grape-derived *p*-coumaric acid and ferulic acid respectively (Singleton 1995, Olsen 1994, Steinke and Paulson 1964). 4-Ethylguaiacol is also a genuine oak component (Chatonnet and Boidron 1989b, Boidron *et al.* 1988, Singleton 1995, Rapp and Versini 1996), but only trace amounts (if any) of 4-ethylphenol are found in oak wood. Nevertheless the concentration of this compound is invariably increased with oak-barrel maturation of red wine as this stage of wine production can sometimes provide ideal conditions for *Brettanomyces / Dekkera* yeast to flourish (Chatonnet *et al.* 1990, Chatonnet *et al.* 1992b, Boidron *et al.* 1988, Singleton 1995, Rapp and Versini 1996).

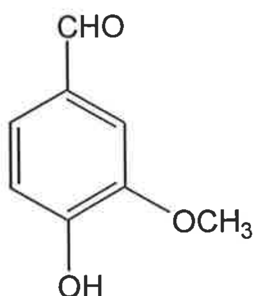
Chatonnet *et al.* (1992b), using the methodology of Boidron *et al.* (1988), determined individual detection thresholds of 605 µg/L and 110 µg/L for 4-ethylphenol and 4-ethylguaiacol respectively in a red wine. However, when combined in a typical ratio of 10:1 the detection threshold is 334 µg/L 4-ethylphenol plus 34 µg/L 4-ethylguaiacol in red wine (Chatonnet *et al.* 1995).
The aroma associated with 4–ethylphenol in red wine has been varyingly described as 'horsy', 'leather', 'medicinal', 'smoky', 'barnyard', 'animal' and 'sweaty-saddle'-like (Boidron *et al.* 1988, Licker *et al.* 1998, Chatonnet *et al.* 1992b, Towey and Waterhouse 1996b). 4–Ethylguaiacol in wine has a smoky, spicy, clove-like aroma (Blank *et al.* 1992, Chatonnet *et al.* 1992b, Holscher *et al.* 1990, Aiken and Noble 1984b, Olsen 1994, Boidron *et al.* 1988). At higher concentrations these odours are undesirable, especially those from 4-ethylphenol (Boidron *et al.* 1988, Towey and Waterhouse 1996b, Licker *et al.* 1998). To some winemakers, even the slightest hint of this character is cause enough to reject a certain wine. To other winemakers and consumers a controlled level is seen to provide an extra dimension of complexity and is even viewed as an integral part of red wine character by many. While 4-ethylphenol and 4-ethylguaiacol are apparently ubiquitous in red wine, their concentrations can vary considerably (Chatonnet *et al.* 1992b and 1995, Licker *et al.* 1999, Pollnitz *et al.* 2000a-c, Chapter 6.2.3).

1.3.4.4 Vanillin, vanillyl alcohol and vanillyl ethyl ether

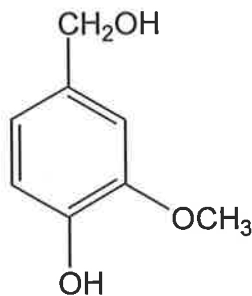
Vanillin is the main aroma component of natural vanilla. This volatile phenol is used widely in the flavour industry (Clark 1990). Vanillin is formed from the thermal degradation of lignin in oak barrels during coopering (eg. Puech 1981, Reazin 1981, Scanlan *et al.* 1968, Schreier 1979) and is then extracted into wines and spirits during their maturation in barrels. There is conflicting opinion in the literature on the importance of vanillin to wine flavour. Threshold data (vanillin aroma perceived as such above 400 μg/L in white wine and 320 μg/L in red wine according to Boidron *et al.* 1988) suggests that vanillin can have a strong influence on wine aroma. Chatonnet *et al.* (1991, 1992a) have concluded that vanillin plays a significant role in the flavour of barrel-aged wines, although this role is much diminished when wines are fermented and stored on lees in oak. Under such conditions, the concentration of vanillin in wine can be reduced by biological and chemical degradation processes (Chatonnet *et al.* 1992a). In contrast, Dubois (1989), citing lower values for typical vanillin concentration in barrel-aged red and white wines and a higher sensory threshold, concluded that vanillin plays no role in the flavour of barrel-aged wines. Dubois (1989) considered the perception of the 'vanilla-oak' character in wines to be due to the influence of oak components other than vanillin. Generalisations on the sensory impact of vanillin in wines should be treated with caution. Barrel-toast levels, maturation time, the presence or absence of microbial activity in wines placed in wood, and perhaps also the strain of organism carrying out the primary and / or secondary fermentations might all have a profound influence on the final concentration of vanillin in a wine. Furthermore, the sensory impact of vanillin is not only likely to vary between individuals (Powers and Shinholser 1988), but may also depend on the presence of other wine components which could modify, mask or enhance its aroma and taste properties.

In a recent sensory study of red and white wines (Spillman *et al.* 1997), the concentration of vanillin in the white wines was positively correlated with 'smoky' and 'cinnamon' descriptors ($p<0.05$ and 0.01 respectively), but was only loosely associated with 'vanilla' ($p<0.10$). In the red wines, vanillin was associated with the descriptor 'vanilla' ($p<0.05$), but was most strongly associated with the descriptor 'coffee'

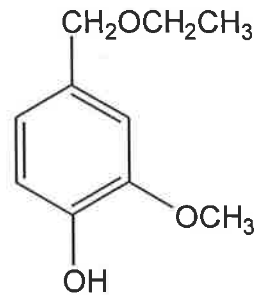
($p<0.001$), and also with 'dark chocolate' and 'smoky' ($p<0.01$). The descriptor 'vanilla' in the red wines was most strongly correlated with the concentration of *cis*-oak lactone ($p<0.001$).



vanillin



vanillyl alcohol

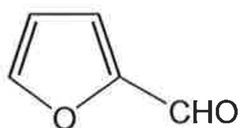


vanillyl ethyl ether

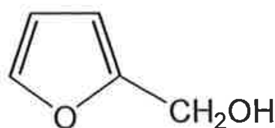
The sensory impact of vanillin will be diminished by transformation to other products during fermentation and maturation of the wine in the barrel. Amongst other products, vanillyl alcohol and its corresponding ethyl ester, vanillyl ethyl ether are formed (Spillman *et al.* 1998a). Vanillyl alcohol and vanillyl ethyl ether have no sensory significance of their own. Measurement of these compounds versus vanillin could be important in estimating the total proportion of extractable vanillin that is converted to other products. Using the method described in this thesis, Osicka (1997) showed a greater than 96% reduction of vanillin to vanillyl alcohol during wine fermentation, followed by a rapid increase in the rate of vanillin extraction thereafter, during maturation over lees.

1.3.4.5 Furfural, furfuryl alcohol and furfuryl ethyl ether

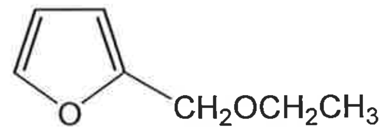
Furfural (2–furaldehyde) has a pleasant wood, caramel or vanilla-like odour (Reazin 1981) and is derived from carbohydrate degradation during the toasting of oak wood and extracted into wines and spirits during maturation in charred oak barrels (eg. Towey and Waterhouse 1996b, Baldwin *et al.* 1967, Reazin 1981, Nishimura *et al.* 1983, Spillman *et al.* 1998a, Schreier 1979, Hodge 1967). Above concentrations of 65 000 µg/L in white wine or 20 000 µg/L in red wine furfural is perceivable as an almond-like aroma (Boidron *et al.* 1988). Furfural has a flavour threshold of 40 µg/L in water (Rothe 1988) and 5 800 µg/L in artificial whisky (Salo *et al.* 1972). In beer at 1000 µg/L, it has a 'sweetish' aroma (Harayama *et al.* 1995). Opinion on the sensory impact in wine is largely based on threshold data in unwooded wines, and does not take into account the possibility of sensory interactions with other volatiles derived from oak or from microbial activity during the maturation of wine. The threshold data alone suggests, that at the concentration range typically found in wine, furfural has, on its own, no more than a minor impact. Furfural, however, has been found to affect the perceived aroma of oak lactone in whisky and Reazin (1981) postulated that these two congeners have an important role in wine and whisky quality. The concentration of furfural and other oak-derived aromatic aldehydes (and, indirectly, their reduction products) in barrel-aged wines depends, *inter alia*, on coopering heat (Chatonnet and Boidron 1989a). Guymon and Crowell (1968) employed paper and thin layer chromatography to separate the aromatic aldehyde fraction of extracts of brandy aged in French and American oak barrels. Although their data for vanillin and syringealdehyde is clearly tabulated, no data is shown for furfural and yet they report that American oak had more furfural than did Limousin oak from France.



furfural



furfuryl alcohol



furfuryl ethyl ether

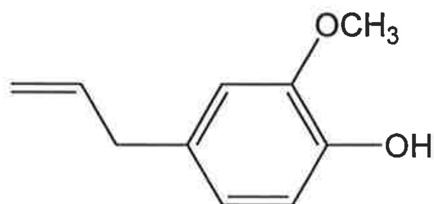
The sensory impact of furfural will be diminished by transformation to other products during maturation of the wine in the barrel. In wine, furfuryl alcohol is formed by

enzymatic reduction of furfural during fermentation and ageing in oak barrels (eg Chatonnet *et al.* 1992a). Furfural levels decrease significantly during the second year of ageing in barrels (Towey and Waterhouse 1996b). Furfuryl alcohol is formed in wine mostly, if not entirely, by this mechanism (Boidron *et al.* 1988, Chatonnet *et al.* 1992a) although the organoleptic role (and fate) of furfuryl alcohol in wine is not well understood. Furfuryl alcohol can be perceived as a 'musty hay' aroma above 35 000 μg/L in white wine and 45 000 μg/L in red wine (Boidron *et al.* 1988).

The corresponding furfuryl ethyl ether has a kerosene like aroma in wine and a sensory threshold of 430 μg/L in white wine (Spillman *et al.* 1998a). Of the 64 barrel-aged red, white and model wines studied by these authors, the highest concentration of furfuryl ethyl ether found was 230 μg/L, which is just over half the sensory threshold.

Schreier (1979) postulated that "furfural present in aged wines does not originate exclusively from the oak casks, but can also be formed from wines during the ageing period" and made the connection that furfuryl ethyl ether was detected in aged wines. Furfuryl ethyl ether can be formed in beer from furfuryl acetate, a presumed fermentation product (Harayama *et al.* 1995, Hayase *et al.* 1996). These authors concluded that the ether was formed via furfuryl alcohol, which was in turn derived from hydrolysis of the acetate. From the data reported in their paper, however, one could also conclude that furfuryl ethyl ether is formed directly from the acetate, via solvation of the furfuryl carbocation. Harayama *et al.* (1995), Hayase *et al.* (1996) found that furfuryl ethyl ether is generated in beer or 5% ethanol solution upon storage due to the reaction between furfuryl acetate and ethanol, and that furfuryl ethyl ether can influence the flavour of beer, giving it a stale aroma. However, Harayama *et al.* (1995), Hayase *et al.* (1996) found that fresh beer spiked with furfuryl ethyl ether (6 ppb) alone did not adequately reproduce the stale aroma, but a combined addition of furfuryl ethyl ether (6 ppb) and (E)-2-nonenal (0.3 ppb) was "strongly stale." (E)-2-nonenal has also been recently reported as an oak compound (see 1.3.4.7).

1.3.4.6 Eugenol



Eugenol (4-allylguaiacol or 4-allyl-2-methoxyphenol) was first isolated from the flower bud of *Eugenia caryophyllata.* It is the main component (70–90%) of the essential oil from cloves (Rothe 1988). Eugenol is present in untoasted oak as a natural product (Chatonnet and Dubourdieu 1998a) and found in wine aged in toasted oak casks (Boidron *et al.* 1988). Eugenol is discernible as a spicy, clove-like aroma at concentrations above 100 μg/L in white wine and 500 μg/L in red wine (Boidron *et al.* 1988).

1.3.4.7 (E)-2-Nonenal, (E)-2-octenal, 1-decanal and 1-hexanal

(E)-2-nonenal

1-decanal

(E)-2-octenal

1-hexanal

Chatonnet and Dubourdieu (1998b) identified the carbonyl compounds (E)-2-nonenal, (E)-2-octenal, 1-decanal and 1-hexanal, all of which had the subtle rancid, weedy odour of 'sawdust', using GC/sniff and GC/MS analyses of extracts of wine matured in new oak barrels. Of these four compounds, (E)-2-nonenal had the most pungent aroma and 1-hexanal the least pungent. 1-Hexanal was rarely present in the wines, but the other three compounds were detected very frequently. Sensory evaluation has shown (E)-2-nonenal can be responsible for 'cardboard-like' and 'stable-like' flavour in beer, and stale flavour when combined with furfuryl ethyl ether (Harayama *et al.* 1995, Hayase *et al.* 1996).

1.3.5 Summary

Oak products can affect wine aroma and flavour – either as barrels or as a cork closure. Physicochemical changes are brought about during the storage of wine in barrels and the contact of bottled wine with corks. Many of these changes produce important effects, evident to the consumer. There are both positive and negative influences on wine aroma and flavour. Proper oak barrel maturation enhances the wine, but bad corks can taint wine. The knowledge of the existence of 'cork taint' is not universal, but consumers and winemakers are becoming aware of the problem.

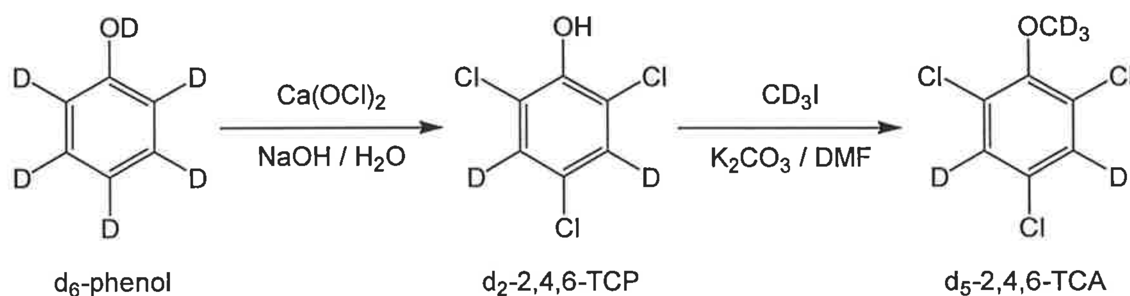
Wine is one of the few consumable commodities whose value increases with age and indeed certain bottle ageing flavour characteristics are due to compounds derived from oak barrel maturation. Anecdotal observation suggests that some of these compounds, such as the oak lactone responsible for 'coconut' aroma in wine, increase with bottle ageing long after the wines barrel ageing was completed. This implies that certain bound flavour precursors are hydrolysed into the key aroma compounds over time. Likewise there can be a decrease in the concentration of other pleasant oakwood derived aromas, as they are converted into derivatives of less aroma or flavour impact. There may also be a potential for tainted corks to impart more taint into the wine during bottle ageing, at least until equilibrium between cork and wine is reached. Better understanding of these changes, their causes and effects will yield an overall improvement in the quality of wine through greater control of the winemaking and storage processes. The development of fast, accurate analytical methods, which can be used to acquire accurate data from which to draw meaningful conclusions, will enhance such understanding.

Chapter 2

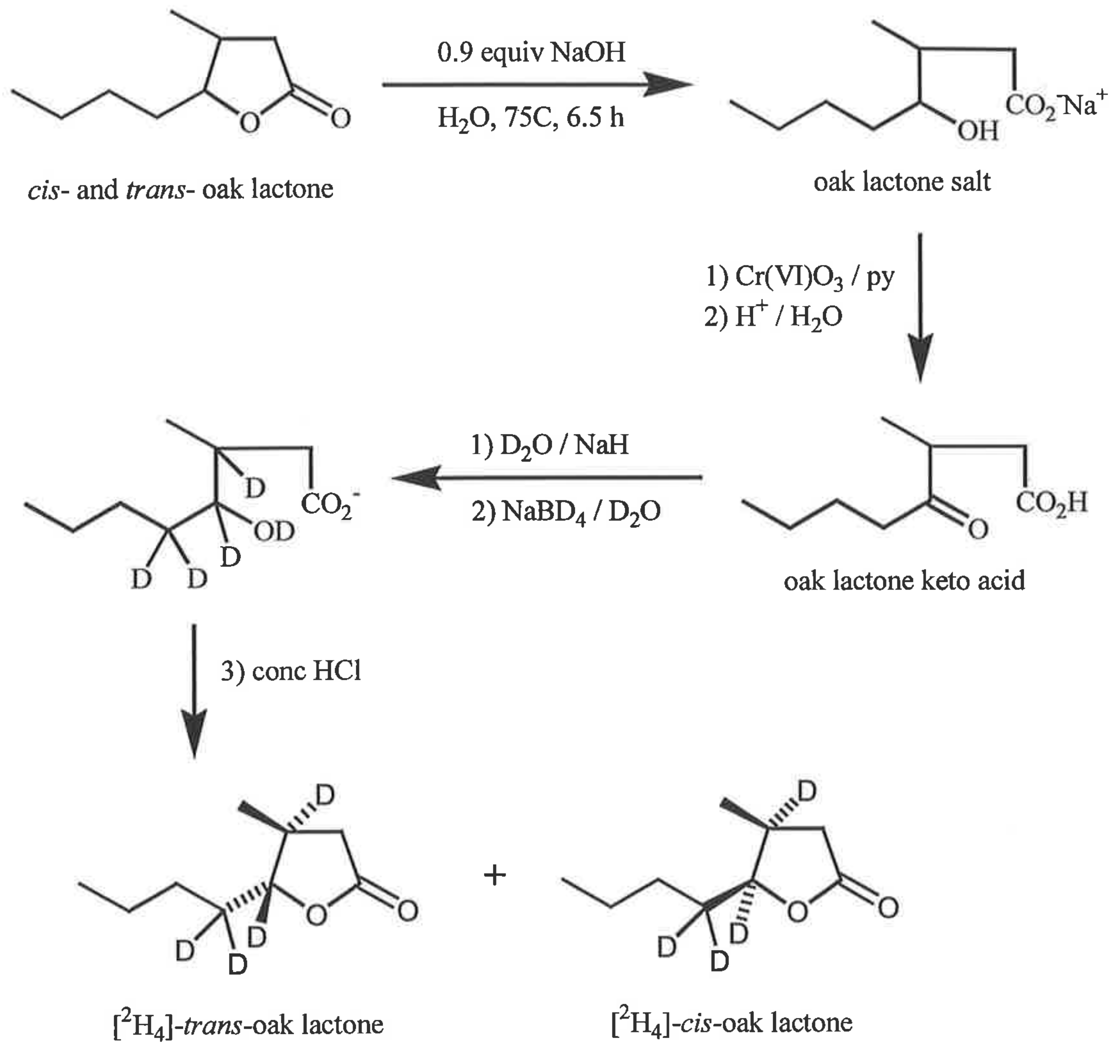
Synthesis of deuterium labelled standards

The labelled standards were prepared as shown in schemes 2.1 – 2.5. The labelled oak lactone isomers were prepared by the author alone, while d_5-trichloroanisole, d_3-vanillin and d_3-guaiacol were prepared by the author in collaboration with Dr George Skouroumounis, Kevin Pardon and Dimitra Capone.

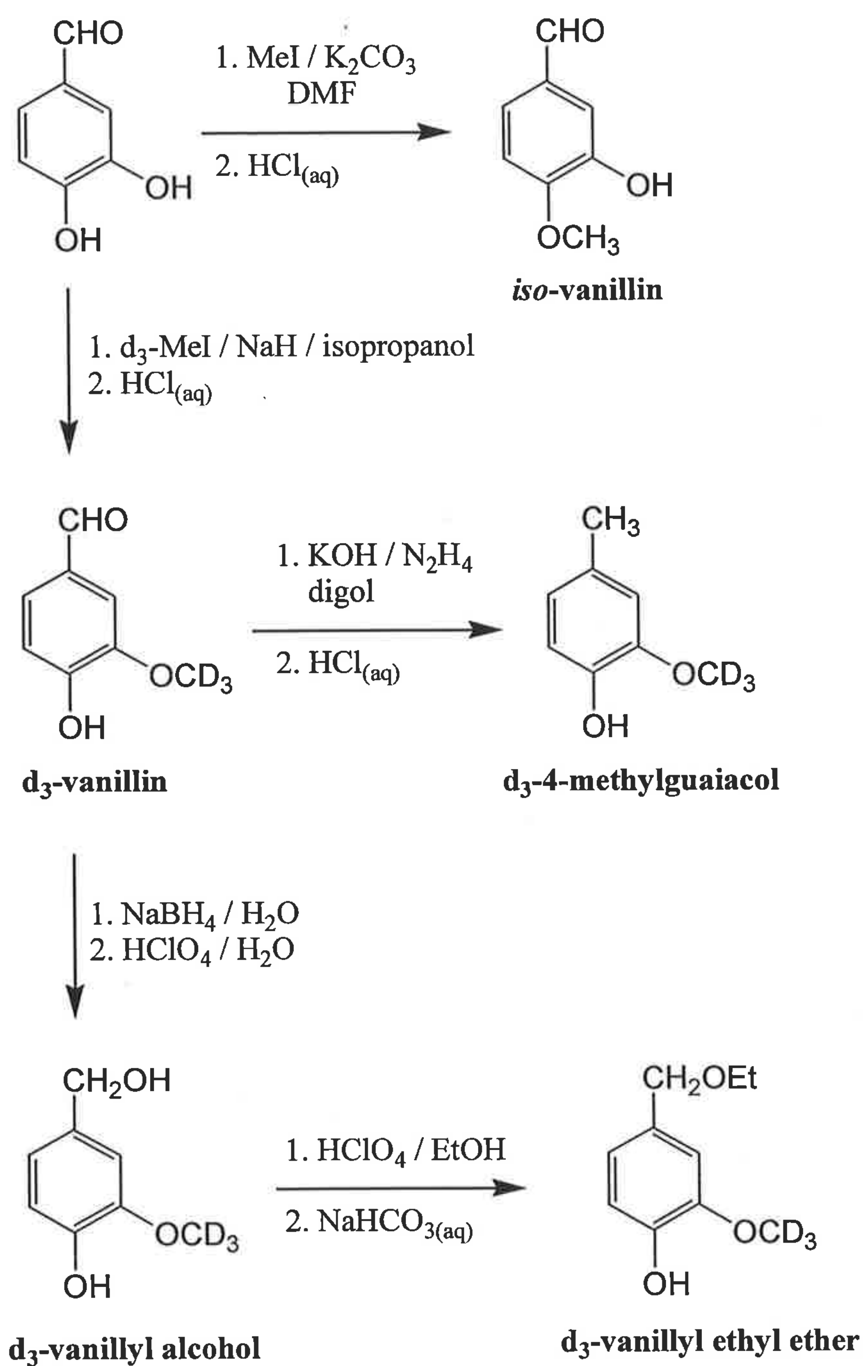
d_3-4-Methylguaiacol, d_4-4-ethylphenol, d_3-vanillyl alcohol and d_3-vanillyl ethyl ether were prepared by others in this laboratory. The syntheses of three of these compounds have now been reported in the literature (Spillman *et al.* 1998a, Pollnitz *et al.* 2000a) and accordingly are not discussed further here.



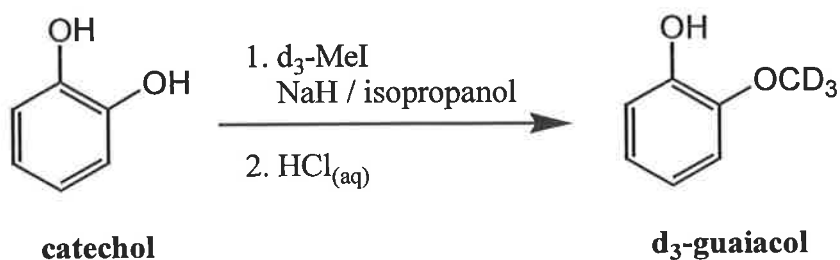
Scheme 2.1 – Preparation of [$^2H_5$]-2,4,6-trichloroanisole (ie. d_5-TCA)



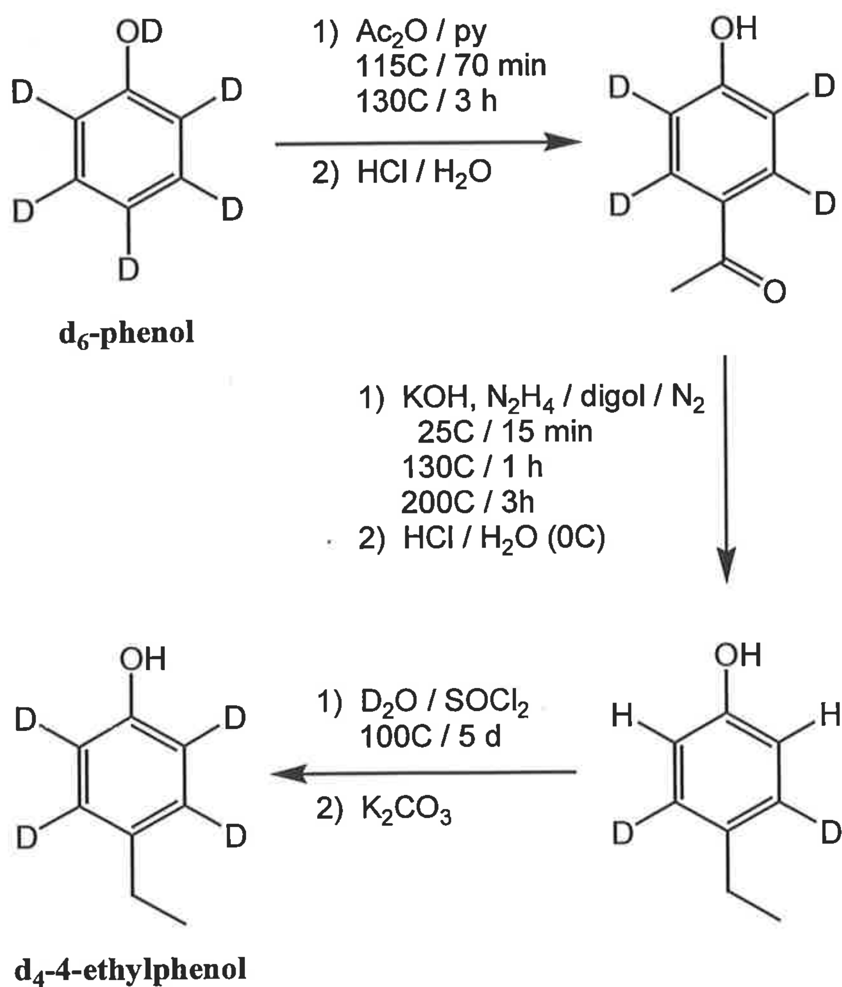
Scheme 2.2 - Synthesis of d_4-*cis*-oak lactone and d_4-*trans*-oak lactone



Scheme 2.3 – Synthesis of d_3-vanillin and derivatives



Scheme 2.4 Synthesis of d_3-guaiacol



Scheme 2.5 Formation of d_4-4-ethylphenol

2.1 Materials and Methods

3,5-[$^2H_2$]-2,4,6-Trichlorophenol (ie. d_2-TCP). $^2H_6$-Phenol (100 mg, Aldrich, 98% $^2H_6$), calcium hypochlorite (1.08 g), 0.1 M aqueous sodium hydroxide (10 mL) and water (20 mL) were stirred at 25°C for 18 h in a closed vessel. A solution of sodium thiosulphate (804 mg) in water (10 mL) was then added to the mixture, followed by 0.45 M phosphoric acid (9.5 mL), bringing the pH to between 3.5 and 4.0. The mixture was extracted with diethyl ether (3 x 30 mL), then dichloromethane (30 mL) and the combined organic extracts were washed with a saturated aqueous solution of sodium hydrogen carbonate (10 mL), followed by saturated aqueous sodium chloride (2 x 10 mL), then dried (anhydrous sodium sulfate). Removal of the solvent gave crude $^2H_2$-2,4,6-trichlorophenol (180 mg, 90% yield) which was recrystallised from *iso*-octane to give large colourless needles (162 mg, 81% yield, one peak on GC/MS). Mass spectrum: *m/z* 202, 200, 198 (M+, 30%, 89%, 100%), 162 (14%), 136 (23%), 134 (37%), 99 (67%).

[$^2H_5$]-2,4,6-Trichloroanisole $^2H_3$-Iodomethane (57 µL) was added to a mixture of 3,5-[$^2H_2$]-2,4,6-trichlorophenol (121 mg) and potassium carbonate (126 mg) in dimethylformamide (1.2 mL) at 25°C under nitrogen, and the mixture stirred for 2 h. Work-up with diethyl ether (sodium carbonate washings) gave $^2H_5$-2,4,6-trichloroanisole (120 mg, 91% yield) as a white solid which was recrystallised from methanol (110 mg, 83% yield, one peak on GC/MS). Mass spectrum: *m/z* 219, 217, 215 (M+, 25%, 80%, 85%), 201, 199, 197 (M-CD_3, 30%, 95%, 100%), 171 (39%), 169 (41%).

4-Hydroxy-3-methyloctanoic acid, sodium salt. A racemic mixture of *cis*- plus *trans*-oak lactone (Allied Flavours, Allied Mills Industries Pty. Ltd., 20 g) was added to aqueous sodium hydroxide (18 mL, 6.5 M, 0.9 molar equiv.) and stirred at 75°C for 6.5 hours under nitrogen with the addition of water (80 mL) after the first hour. The reaction mixture was then washed with diethyl ether (2 x 50 mL) to remove unreacted oak lactone, concentrated, and dried to constant weight *in vacuo* to yield the sodium salt of 4-hydroxy-3-methyloctanoic acid as a white powder (22.6 g).

Cis- *plus* trans-*4,5-[$^2H_2$]-5-(1,1-[$^2H_2$]-butyl)-4-methyl-4,5-dihydro-2(3H)-furanone (ie d_4*-cis- *plus d_4-trans-oak lactone)* Chromium (VI) trioxide (13.4 g, 99.9% Aldrich) was added to a solution of the sodium salt (5 g) of 4-hydroxy-3-methyloctanoic acid in anhydrous pyridine (*circa* 125 mL) and stirred overnight at 25°C. The reaction mixture was cooled to 0°C and acidified to pH<1 with dilute hydrochloric acid. The sodium hydrogencarbonate-soluble organic fraction of the crude product was isolated with ethyl acetate by liquid-liquid extraction and distilled *in vacuo* with a Kugelrohr distillation apparatus to yield 3-methyl-4-oxo-octanoic acid (2.1 g) as a clear liquid, *m/z* 172 (M^+, 2%), 130 (38%), 115 (17%), 112 (17%), 103 (13%), 87 (13%), 85 (100%), 57 (74%). Several batches of the keto acid were prepared in this way.

Sodium hydride (0.6 g, dry, 95%, Aldrich), then a solution of 3-methyl-4-oxo-octanoic acid (2.73 g) in deuterium oxide (10 mL), was added to deuterium oxide (30 mL) and stirred at room temperature under nitrogen overnight. Sodium borodeuteride (3.0 g, 98 atom % ^2H, Aldrich) and deuterium oxide (20 mL) were then added and the reaction mixture was stirred at room temperature under nitrogen for a further 24 h. before quenching to pH<1 with aqueous hydrogen chloride (32%, 10 M) and stirring for an additional 24 h. The crude product was isolated with diethyl ether and purified by column chromatography on silica gel 60 using hexane-diethyl ether (3:1) as eluant, yielding pure d_4-*trans*-oak lactone (85 mg), a mixture of d_4-*cis*- and d_4-*trans*-oak lactone (1.31 g), and pure d_4-*cis*–oak lactone (53 mg). All fractions were redistilled *in vacuo* to give clear liquids. d_4-*Trans*-oak lactone, *m/z* 160 (M^+, 3%), 132 (4%), 118 (6%), 102 (6.5%), 101 (100%), 90 (13%), 72 (13%), 71 (6%).

d_4-*Cis*-oak lactone, *m/z* 160 (M^+, 3%), 132 (4%), 118 (6%), 102 (6.5%), 101 (100%), 90 (21%), 72 (21%), 71 (10%). No *m/z* 159 or 157 ions were detected in the mass spectra of the lactone isomers. A small *m/z* 158 ion in the spectra was attributed to loss of deuterium from the molecular ion, associated with fragmentation of the C_5-H bond (furanone numbering). Analysis, by GC/MS, of a mixture of weighed aliquots of the pure isomers was used to determine the composition of the major (mixed) fraction obtained from the synthesis. This major fraction was used for all analyses of oak lactone in the wine and oak extracts described in later chapters.

4-Hydroxy-3-([$^2H_3$]-methoxy)-benzaldehyde (ie. d_3-vanillin). 4-Methyl-2,6-di-t-butylphenol (232 mg), followed by 3,4-dihydroxybenzaldehyde (5.01 g), then [$^2H_3$]-methyl iodide (4 mL), was added to a solution of sodium hydride (2.49 g) in *iso*-propanol (200 mL) under nitrogen. The mixture was stirred at 25°C for 17 h, then acidified with conc. aqueous hydrogen chloride to pH < 7 and evaporated to dryness *in vacuo*. The product was dissolved in diethyl ether (200 mL) and washed with water (3 x 10 mL), 10% aqueous sodium bisulfite (3 x 1 mL) and a solution of 15 drops of sat. sodium bicarbonate in sat. aqueous sodium chloride (5 mL). The ethereal extract was dried (anhydrous magnesium sulfate) and the ether evaporated giving crude product (6.08 g), which was purified by dry column chromatography using 230-400 mesh silica as the stationary phase and dichloromethane as the eluant. Column fractions were washed with aqueous sodium bisulfite and dried (anhydrous magnesium sulfate). Removal of the solvent and recrystallisation of the major product from ethyl acetate / hexane gave d_3-vanillin (3.2 g, 60% yield, purity > 99% by GC/MS) as white crystals. Mass spectrum: *m/z* 155 (M^+, 95%), 154 (100%), 126 (11%), 109 (10%), similar to that reported by Semmelroch *et al.* (1995).

2-Methoxy-[$^2H_3$]-4-methylphenol (ie. d_3-4-methylguaiacol) was prepared by Mr Kevin Pardon in the following way. A solution of [$^2H_3$]-vanillin (500 mg) (as made above in Scheme 2.3), potassium hydroxide (1.24 g, 15 molar eq.) and hydrazine hydrate (>99% pure, 0.54 mL, 7.6 molar eq.) 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 isolated with *n*-pentane (6 x 30 mL). The product was purified by distillation under reduced pressure with a Kugelrohr microdistillation apparatus to yield [$^2H_3$]-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%).

[$^2H_3$]-2-Methoxyphenol (ie. 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 hydride (2.52 g) in *iso*-propanol (320 mL) under nitrogen. The mixture was stirred at 25°C overnight, then acidified with conc. 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 x 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 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 crystallised 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 (1993).

2.2 DISCUSSION

[$^2H_5$]-2,4,6-Trichloroanisole (Scheme 2.1)

Deuterium-labelled phenol (d_6-phenol) was chlorinated with excess calcium hypochlorite at room temperature under basic conditions giving d_2-2,4,6-trichlorophenol (d_2-2,4,6-TCP) as the major product in 81% yield.

Even under prolonged reaction times at room temperature, little additional chlorination took place. Only traces of tetrachlorophenol and no pentachlorophenol were formed (GC/MS analysis – data not shown). The d_2-TCP was methylated with d_3-iodomethane to form d_5-2,4,6-trichloroanisole in 83% yield.

d_4*-Cis-*oak lactone and* *d_4*-trans-*oak lactone (Scheme 2.2)

Deuterium-labelled isomers of *cis*- and *trans*-oak lactone were prepared from a racemic mixture of unlabelled *cis*- and *trans*-oak lactone as shown in Scheme 2.2. Deuterium exchange alpha to the keto group was done under strongly basic conditions at room temperature. Back-exchange of deuterium from the keto-acid was avoided by carrying out the reduction of the keto group with sodium borodeuteride *in situ*. The racemic mixture of d_4-*cis*- and d_4-*trans*-oak lactone was purified by column chromatography to give fractions of 100% *cis*-oak lactone, 100% *trans*-oak lactone and mixtures of both.

d_3-Vanillin and d_3-4-methylguaiacol (Scheme 2.3) d_3-Methylation of the dianion of 3,4-dihydroxybenzaldehyde took place mainly on the less stable and therefore more reactive phenoxide anion meta to the aldehyde group, giving d_3-vanillin in 60% yield (after purification). When the reaction was carried out using standard conditions for methylating phenols (methyl iodide and potassium carbonate in dimethylformamide) *iso*-vanillin was the major product. d_3-Vanillin has also been prepared before by Semmelroch *et al.* (1995), who used 3,4-dihydroxybenzaldehyde, d_6-dimethylsulphate and cite the methodology of Schwyzer (1930). These authors quoted no yield.
d_3-4-Methylguaiacol was prepared from d_3-vanillin under standard Wolff-Kischner reduction conditions.

d_3-Guaiacol (Scheme 2.4) was synthesised in 30% yield (purified product) by methylation of catechol with 1.0 equiv. of d_3-methyl iodide under strongly basic conditions (sodium hydride in isopropanol). d_3-Guaiacol has been prepared previously by Cerny and Grosch (1993) who reacted d_6-dimethylsulphate with a solution of catechol dissolved in nitrobenzene under basic conditions which were maintained by careful monitoring and additions of aqueous sodium hydroxide to keep the pH between 8 and 9 for 1 hour, after which they acidified the mixture, extracted three times with ether then extracted the combined ether portions with aqueous sodium hydroxide, twice, then reacidified the aqueous layer and extracted that three times with ether and then purified the [$^2H_3$]-guaiacol via thin layer chromatography. This latter method appears to be unnecessarily convoluted and no yield was quoted.

Chapter 3

Quantitative analysis of chloroanisoles

In this chapter, a new method is developed and validated for the analysis of 2,4-dichloroanisole (2,4-DCA), 2,6-dichloroanisole (2,6-DCA), 2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA) and pentachloroanisole (PCA) in wine and cork extracts versus polydeuterated TCA as internal standard. The method is fast, accurate, precise, sensitive and suitable to the analysis of large numbers of samples.

3.1 MATERIALS AND METHODS

Preparation of samples for analysis

a) Wine. $^2H_5$-2,4,6-Trichloroanisole (10 ng) in ethanol (100 µL) was added to a wine sample (200 mL). After thorough mixing (eg. vigourous shaking with inversion and swirling for 10 seconds), the sample was extracted with redistilled *n*-pentane (3 x 4 mL). The pentane extracts were combined, spectroscopic grade *iso*-octane (2,2,4-trimethyl pentane, Spectrosol, APS Ajax Finechem) (~3 drops) was added and the solution concentrated to about 50 µL by fractional distillation through a Vigreux column packed with Fenske helices, and further concentrated under a stream of nitrogen at room temperature immediately prior to instrumental analysis.

The *iso*-octane (b.p. 99.3°C), added to all the pentane extracts, minimises the loss of chloroanisoles in the concentration steps, and, combined with the initial oven temperature of 60°C, focuses the solvent band on the injector end of the GC column thus giving sharper peaks.

b) Whole Cork. Whole corks were immersed in redistilled *n*-pentane (37 mL) in a stoppered vessel for 48 h, after which the cork was removed. $^2H_5$-2,4,6-Trichloroanisole (50 ng) in *n*-pentane (1 mL) and spectroscopic grade *iso*-octane (2,2,4-trimethyl pentane) (~3 drops) were then added and the extract was concentrated as described above.

c) Ground Cork. After weighing, each cork was ground with a Krups KM75 160W coffee grinder, transferred to an all-glass vessel and soaked in redistilled *n*-pentane (37 mL) for 24 h. To avoid cross contamination, between the grinding of each cork portion, the coffee grinder was cleaned meticulously with at least three rinses each of redistilled

dichloromethane and redistilled *n*-pentane, alternately, and a final rinse with redistilled *n*-pentane. The ground cork was removed by filtration through a small plug of glass wool at the base of a Pasteur pipette. Internal standard, $^{2}H_{5}$-2,4,6-trichloroanisole (500 µL of a 100 ng/mL solution in redistilled *n*-pentane) and spectroscopic grade *iso*-octane (2,2,4-trimethyl pentane) (~3 drops) were added, and the extract was concentrated as described above.

Instrumental analyses

Extracts were analysed with a Hewlett-Packard 5890A series II gas chromatograph coupled to a Hewlett-Packard 5971 mass spectrometer (GC/MS). The gas chromatograph was fitted with an approx. 30 m x 0.25 mm J&W fused silica capillary column DB-1701, 0.25 µm film thickness. The oven temperature was started at 60°C, held at this temperature for 2 min, then increased to 250°C at 10°C/min and held at this temperature for 20 min. The injector was held at 220°C and the transfer line at 275°C. The sample volume injected was 3 µL. The splitter, at 30:1, was opened after 36 sec. The liner used was resilanised borosilicate glass, tapered at the column interface, with a plug (2-4 mm) of resilanised glass wool positioned just above the taper. The residence time for the needle in the injector block was approx. 100 ms. Positive ion electron impact spectra at 70 eV were recorded in the range *m/z* 35-450 for scan runs. Unlabelled chloroanisole reference standards were purchased from Aldrich Chemical Company Inc. Separate solutions (1 mg / mL) of each compound were analysed in scan mode to determine retention times, elution profile and mass spectrum of each chloroanisole, individually. For quantification, selected fragment ions were monitored for 50 ms each in the Selective Ion Monitoring (SIM) runs. The ions monitored were: *m/z* 161, <u>176</u>, 178 for 2,4-dichloroanisole and 2,6-dichloroanisole; *m/z* 195, <u>210</u>, 212 for 2,4,6-trichloroanisole; *m/z* <u>215</u>, 217 for d_5-2,4,6-trichloroanisole (Internal Standard); *m/z* 203, 229, <u>246</u> for 2,3,4,6-tetrachloroanisole; *m/z* 237, 265, <u>280</u> for pentachloroanisole. The underlined ions were the ones usually used for quantitation (by peak area), however occasionally another ion was used for quantitation to avoid interference by co-eluting peaks in the target ion. (Avoidance of co-eluters is discussed in more detail in section 4.2.1.1).

Validation of the analytical method

Aliquots (200 mL) of commercial red wine (4L "bag-in-box" or "cask") were spiked with a solution of 2,4-DCA, 2,6-DCA, TCA, TeCA and PCA to give concentrations of 0 ng/L, 2 ng/L, 5 ng/L, 10 ng/L, 20 ng/L and 50 ng/L. Duplicate spiked solutions were prepared at each concentration, except at 2 ng/L where five replicates were prepared. Further spiked additions to aliquots (200 mL) of another commercial red wine (20L "bag-in-box") and a commercial white wine (20L "bag-in-box") were prepared to give concentrations of 0.5 ng/L, 1 ng/L, 2 ng/L, 5 ng/L, 10 ng/L, 20 ng/L, 50 ng/L, 100 ng/L, 250 ng/L and 500 ng/L. Duplicate spiked solutions were prepared for each wine at each concentration, except for the red at 500 ng/L where five replicates were prepared. All these spiked wines were extracted and analysed to generate calibration curves that were linear throughout their concentration ranges for all five chloroanisoles (see results and discussion below).

3.2 RESULTS AND DISCUSSION

As discussed in chapter 1.2.1, stable isotope labelled analogues of analytes are virtually identical chemically to the analytes themselves. Thus, the accuracy of the analysis is not reduced by inefficiency in isolation, by analyte decomposition or by losses due to evaporation during concentration. As expected, standard additions experiments showed that by using d_5-2,4,6-trichloroanisole as an internal standard, TCA in wine extracts could be measured with a high degree of precision and accuracy (coefficient of determination, $r^2 = 0.9998$).

The chemical stability of the deuterium labels on d_5-TCA during wine storage was demonstrated by an experiment in which a model wine solution of d_5-TCA was heated to 55°C at pH 3.0 for two months. Mass spectrometry showed that no loss of deuterium from the d_5-TCA occurred over that period.

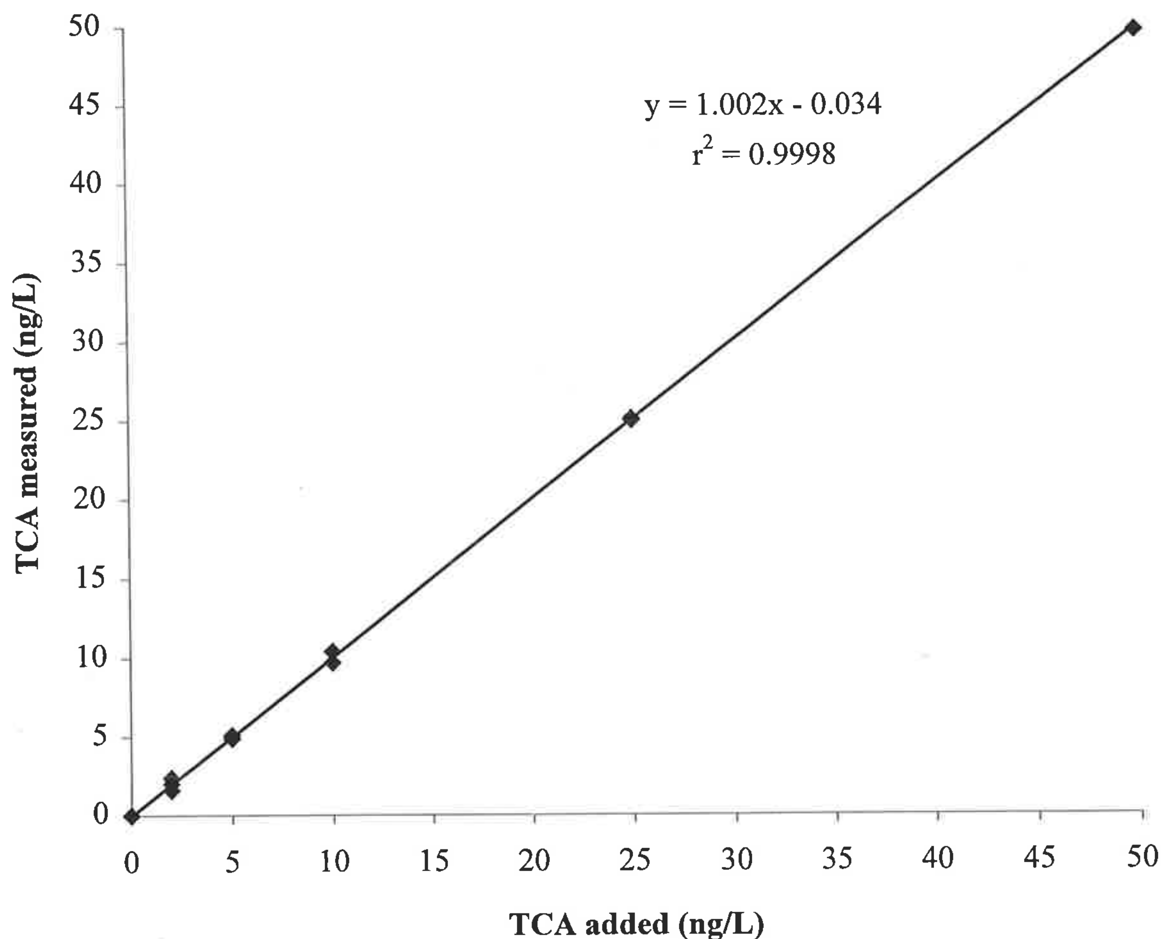


Figure 3.1 Calibration curve generated from standard additions of TCA to a red wine

Note that the points plotted are duplicate points for each concentration, except for the 2 ng/L spike level, which has five points.

Although not a SIDA, good precision and accuracy were nevertheless also obtained for standard additions of the four other chloroanisoles thought to be commonly associated with cork taint, *viz.* 2,4-DCA, 2,6-DCA, 2,3,4,6-TeCA and PCA when d_5-TCA was used as the internal standard. As the chloroanisoles other than TCA differ from d_5-TCA in their volatility, care needs to be taken during the concentration step (eg. under no circumstances should the extract be allowed to concentrate to dryness). Similar standard curves to TCA (Fig 3.1 above) were obtained for each of these four common chloroanisoles in the white and red wines. All the curves were linear throughout the concentration range. Their correlation coefficients (r^2) are shown in table 3.1.

Table 3.1

Correlation coefficients (r^2) for chloroanisole standard addition curves in red and white wines

	red wine r^2 0-50 ng/L	red wine r^2 0-500 ng/L	white wine r^2 0-500 ng/L
2,6-DCA	0.986	0.996	0.956
2,4-DCA	0.980	0.997	0.995
2,4,6-TCA	1.000	0.999	1.000
2,3,4,6-TeCA	1.000	1.000	0.997
PCA	0.991	0.999	0.997

There was some interference by co-eluting peaks for the early eluting dichloroanisoles, which is why their correlation coefficients are not as high as those for the other chloroanisoles.

The lower quantitation limit (with qualifier ions to confirm identity) in wines and corks varied between 0.1 and 2 ppt, depending on the concentration of the final pentane solution, interference from other wine or cork components and background noise in the mass spectrometer. At low concentrations, the precision of the analysis depended largely on the signal to noise ratio. Analysis of five replicates of a red wine spiked with 2 ng/L of TCA gave a mean concentration of 1.9 ng/L, with a standard deviation of 0.33 ng/L. Adequate repeatabilities were obtained for the other four chloroanisoles (Table 3.2). However as these compounds differ from TCA, and thus d_5-TCA, in their volatility, care needs to be taken during the concentration step. Indeed, for these four compounds, good reproducibility could not always be obtained when the same concentrated pentane extracts were diluted with a small amount of pentane for storage, then re-concentrated under a stream of nitrogen and analysed a second time. Accuracy and repeatability for the analysis of each of the five common chloroanisoles at the higher spike level of 500 ng/L (Table 3.3) was much better than that of the lower level.

Table 3.2

Five replicate analyses at the 2 ng/L spike level, ng/L measured in red wine

	2,6-DCA	2,4-DCA	TCA	TeCA	PCA
Mean	2.2	2.0	1.9	1.7	2.0
SD	0.95	0.18	0.33	0.52	0.05
SD/Mean	43%	9%	17%	30%	2.5%

Table 3.3

Five replicate analyses at the 500 ng/L spike level, ng/L measured in red wine

	2,6-DCA	2,4-DCA	TCA	TeCA	PCA
Mean	500	500	500	500	500
SD	28	20	14	9.6	12
SD/Mean	5.6%	4.0%	2.8%	1.9%	2.5%

No repeatability or standard curve data could be obtained in a cork matrix because there is no satisfactory way of spiking the cork with unlabelled chloroanisoles, the analysis is destructive, no two corks are exactly the same, there is no way of telling (prior to destructive analysis) the level of unlabelled chloroanisoles within the cork, and incurred distribution of any chloroanisoles throughout the cork is not uniform (Chapter 5, Pollnitz *et al.* 1996, Howland 1997, Howland *et al.* 1997).

Capone *et al.* (1999) re-extracted ground corks by the method and observed that each successive extract contained about one third of the TCA of the previous extract and that the sum of TCA in the first two extracts was always more than 90% of the sum of the four successive extracts. In this laboratory, the analysis of two successive extracts has been the standard method for determining the chloroanisole composition of corks. This method is a compromise between accuracy (< 100%, but > 90% of the chloroanisoles are extracted) and efficiency (as four to six extractions are prohibitively time consuming).

Unlike the analysis for oak-derived flavour volatiles (Chapter 4), studies into the artefactual generation of chloroanisoles during the extraction and analysis were not undertaken. This issue was not considered to be problematic as chloroanisoles are very stable compounds formed by biological methylation, rather than being formed from the thermal degradation of precursor forms extracted from the oak and present in the matrix being analysed. For all the chloroanisoles, no artefactual generation with changing injector temperature has been observed over several years of using the analyses.

3.2.1 Alternative methods of analysis for TCA and other chloroanisoles

In comparison to the method developed in this thesis, other methods in the literature are more time consuming, less sensitive or less accurate. The best of them is that employed by Whitfield *et al.* (1986) using 3,5-dimethyl-2,4,6-TCA as an internal standard for their analyses. This standard is structurally similar to TCA and is not found in tainted wines or corks (Chapter 5, Pollnitz *et al.* 1996, Capone *et al.* 1999). Whitfield *et al.* (1986) analysed TCA, TeCA and PCA in foods and packaging materials by GC/MS, but did not measure dichloroanisoles. Whitfield *et al.* (1986) obtained good levels of detection of 0.01 ng/g and could quantitate in the range of 0.1-100 ng/g with a reproducibility of 2 to 11% co-efficient of variance shown in their standard addition curves, prepared in the same matrix as the samples analysed. Their extraction method was time consuming (approx. 1-4 samples per day) and involved a concentration step. Whitfield *et al.* (1986) demonstrated an awareness of mass spectral ratio drift and analysed each extract in triplicate to avoid a "slow, random fluctuation in ion source sensitivity", but with their time consuming extraction method they could not possibly analyse their standard curves and samples under the same instrumental conditions – thus although precision of analyses would be acceptable over a day, the same extracts analysed more than a week apart would most likely give different values within +/- 10% (approx).

Amon *et al.* (1989), using the methodology of Whitfield *et al.* (1986), quantified 2,4,6-TCA in affected wines and corks by GC/MS, obtaining an approximate level of

detection of 1 ppt for TCA. Amon *et al.* (1989) extracted wine (200 mL) with pentane (3 x 4 mL) using the method of Tanner and Zanier (1983), except inverting the volumetric flask and shaking it in a circulatory motion for 2-3 hours (per 4 mL pentane extraction) in a shaking apparatus. This limited the number of extracts they could do to a maximum of several per day. The combined pentane extracts were concentrated on a Vigreux apparatus to approx. 10 μL for manual injection (of 4 μL) into the GC/MS. For cork assays, Amon *et al.* (1989) soaked whole corks in absolute ethanol for 48 hours, then diluted the extract to 200 mL with water and then extracted and analysed the solution by the same method as they did for wine. Their wine analysis was validated by spiked standard addition curves in dry white wine but no correlation data is shown in the paper. They did not show recovery data, nor demonstrate that all the TCA is extracted from wine or cork, but it is evident from their extraction procedure that they did attempt to get complete extraction of the analytes of interest.

In another example, Duncan *et al.* (1997) analysed TCA in cork by grinding it in a hammer mill, followed by two pentane extractions. Their combined pentane extracts were concentrated in a Vigreux column, and analysed by GC/MS. No internal standard was used – instead they halved the combined pentane extract and added unlabelled TCA (125 ng) to one half, and divided their result by the recovery of this one addition – by doing this they have no way to tell if their injection volume from run to run is consistent and thus the accuracy of their quantitation is at the mercy of bubbles in the syringe, temperature variations (ie. vapour pressure of the pentane extract affecting the volume injected), and viscosity of the extracts (which can vary considerably due to different levels of wax and coating on the cork). They showed good repeatability in pure solvent (pentane extracts of 40 +/- 0.7 ng/L TCA), but the validity of extrapolating this to real cork extracts has not been demonstrated. Repeatability in a pure solvent matrix is of little value without due attention to other influencing factors such as interference from other components. Duncan *et al.* (1997) obtained a detection limit of 1 ng/L in their pentane extracts, which translates to approx. 5 ng/L wine. In summary, the method of Duncan *et al.* (1997) is approx. five times less sensitive and more inaccurate than the method of Amon *et al.* (1989).

As a third example, Rocha *et al.* (1996) analysed volatiles including TCA in cork slabs by GC/MS. Their method used 90 g of cork, was time consuming (including a three hour steam distillation / extraction, freezing out the water from the pentane extract, and concentration via a Vigreux column) and inaccurate (ethyl pentanoate as internal standard (IS), assumed equal extraction of all volatiles and IS, assumed equal proportions of IS and all other volatiles lost during concentration, quantitated by total ion chromatogram only (hence at the mercy of any co-eluting peaks) and assumed equal responses for all analytes of interest and IS). They obtained a level of detection of approx. 5 ppt for TCA but did not quote any r^2, accuracy or precision measurements.

Electron capture detection (ECD) coupled to GC is used for the analysis of chloroanisoles (eg Curtis *et al.* 1974a, Gee *et al.* 1974, Häggblom *et al.* 1988), with a level of detection comparable to good mass spectrometers. However unlike mass spectrometry, ECD gives little information (apart from retention time) about the identity of the analyte. ECD can only detect halogenated compounds and has no easy means of discriminating between chlorine and bromine and iodine, or even how many (eg) chlorine atoms are attached to the compound detected. Thus, ECD has no means of determining if two compounds coelute (eg 2,4-DCA and TCA on most columns suited to a wine or cork extract matrix). Hence, GC/ECD is not the method of choice to resolve a mixture of all 19 possible mono- through to penta- chloroanisoles (as done with GC/MS in chapter 5.1.3). ECD also has the potential problem of interference from 'negative peak' substances in some extracts (Gee *et al.* 1974, Whitfield *et al.* 1986).

After the publication of the work described in this chapter (Pollnitz *et al.* 1996), Evans *et al.* (1997), using a sample of d_5-TCA synthesised in this laboratory, analysed TCA in wine by successfully adapting the methodology of Pollnitz *et al.* (1996) to incorporate automated SPME. Evans *et al.* (1997) only obtained a quantitation limit of 5 ng/L with an estimated signal to noise ratio of 8:1, could only analyse wine samples (not corks), and did not measure any other chloroanisoles. Only two ions were monitored, one for TCA and one for d_5-TCA thus there was no confirmation of identity apart from retention time and peak shape. The GC/MS conditions cited in their paper are good for rapid

analyses, but not optimum for MS sensitivity on a Hewlett-Packard 5971 fitted with a 5972 upgrade – the helium gas carrier flow rate is too fast (at 45 cm/s rather than 31 cm/s for a 0.25 mm ID column) and thus introduces too many analytes into the pseudo 5972 MS (which works better at a higher vacuum). Also a higher signal to noise ratio is obtainable at the ng/L level by adding approx. 400 electron multiplier volts (emv) to the default tune value, rather than the 294 emv that they added. Thus, the method of Evans *et al.* (1997) could be improved to obtain better sensitivity or incorporate some qualifier ions. Their extraction method was quick and automated, however, and did not require a concentration/evaporation step. Their repeatability ranged from 5-13% co-efficient of variance for TCA additions to white wine in the concentration range of 10-250 ng/L. Their standard curve, spiked in white wine over the range 0 and 5-250 ng/L (n = 6 x 4) had a coefficient of determination (r^2) of 1.000.

3.3 Conclusion

The method developed in this chapter is fast, accurate, sensitive and precise. Combined with automated instrumental analyses the method has enabled the author to quantitate TCA and other chloroanisoles in large numbers of wine and cork samples. The method has been used in many problem-solving trials for industry, has had relevant research applications (eg. Chapter 5, Pollnitz *et al.* 1996, Howland *et al.* 1997, Howland 1997, Liacopoulos *et al.* 1999, Capone *et al.* 1999, Barker *et al.* 2000),
and is offered as a commercial service to industry
(http://winetitles.com.au/awri/tca.html).

Chapter 4

Quantitative analysis of volatile wine components associated with oak barrel maturation

This chapter deals with the multi oak-component assay that measures guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans* and *cis*-oak lactone and vanillin in the one extraction and GC/MS run.

The analyses of vanillyl alcohol and vanillyl ethyl ether are briefly discussed separately.

4.1 MATERIALS AND METHODS

4.1.1 Quantitative analysis 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 wines or 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 wine sample (5 mL) in a screw cap vial using a glass syringe (100 μL Hamilton). The organic solvent, usually diethyl ether : pentane (1:2, *circa* 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.

For analysis by solid-phase microextraction (SPME), 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) using a glass syringe (100 μL Hamilton), and sodium chloride (*circa* 1 g) were added to the wine (5 mL) in a screw cap vial with a teflon seal. A SPME portable field sampler (Supelco, Bellefonte, USA) fitted with a 65 μm Carbowax-DVB fibre assembly (Supelco, Bellefonte, USA) was used to sample the headspace above the stirred wine sample for 20 minutes at room temperature, immediately prior to instrumental analysis.

Instrumental Analyses

Samples were analysed with either a Hewlett-Packard (HP) 5890A Series II gas chromatograph coupled to a HP 5971 mass spectrometer or with a HP 6890 gas chromatograph coupled to a 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 purge splitless mode. The gas chromatograph was fitted with an approx. 30 m x 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. then increased to 250°C at 10°C/min and held at this temperature for 20 min. The injector was held at 200°C and the transfer line at 280°C. For liquid injections, the sample volume injected was 2 μL, the splitter, at 30:1, was opened after 36 sec. and the liner used was resilanised borosilicate glass, tapered at the column interface, with a plug (2-4 mm) of resilanised glass wool. 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 the Selective Ion Monitoring (SIM) mode. The ions monitored in SIM runs were: *m/z* 81, 109, <u>127</u> for $^2H_3$-guaiacol; *m/z* 81, 109, <u>124</u> for guaiacol; *m/z* 95, 123, <u>141</u> for $^2H_3$-4-methylguaiacol; *m/z* 95, 123, <u>138</u> for 4-methylguaiacol; *m/z* <u>90</u>, <u>101</u>, 118, 132 and 160 for $^2H_4$-*trans* and $^2H_4$-*cis*-oak lactone and *m/z* <u>99</u>, 114, 128 and 156 for *trans*- and *cis*-oak lactone; *m/z* 111, <u>126</u> for $^2H_4$-4-ethylphenol; *m/z* 107, <u>122</u> for 4-ethylphenol; *m/z* 122, 137, <u>152</u> for 4-ethylguaiacol; *m/z* 154, <u>155</u> for $^2H_3$-vanillin and *m/z* 151, <u>152</u> for vanillin. The underlined ions were the ones usually used for quantitation (by peak area), however occasionally another ion was used for quantitation to avoid interference by co-eluting peaks with the target ion. Selected fragment ions were monitored for 50 ms each, except for the oak lactone isomers and their isotopically labelled analogues whose ions were monitored for 25 ms each.

The analyses by SPME were done in the same manner, except as follows; the splitter was opened after 5 min. after which the fibre was baked for at least another 10 min. before retraction; the liner was resilanised borosilicate glass, direct injection (with minimal dead volume); 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 oak volatiles assay was validated by a series of standard addition experiments to red, model and white wine matrices. 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, *cis*-oak lactone, *trans*-oak lactone and vanillin. Red, white and model wines were spiked at 0, 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 μg/L with 4-ethylphenol and 4-ethylguaiacol. All of the spiked samples were prepared, extracted and analysed in duplicate.

Studies on the artefactual generation of oak volatiles during the analysis 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 & B), two of *Quercus alba*-medium grain (A & B), and two of Chestnut Oak (*Q prinus*) (A & 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 the author. About 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. The 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 minimise the loss of any escaping volatiles. All twelve samples of shavings (*circa* 100 g each, but each weighed exactly, masses in Appendix I) were soaked in 1 L of model wine (10% ethanol, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid) 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 & B, heated and unheated) were also extracted and analysed in triplicate by 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 replicate was analysed by the method at three different injector block temperatures; 200°C, 225°C and 250°C, giving a total of thirty determinations for each model wine solution of *Q. alba* fine grain B and Chestnut Oak A & B, heated and unheated. Separate 5 mL aliquots from the remaining six solutions were extracted and analysed in triplicate by SPME and also by liquid-liquid extraction with pentane-diethyl ether (2:1) (~2 mL) and injected at two different injector block temperatures; 200°C and 250°C, giving a total of nine determinations for each model wine solution of *Q. Alba* fine grain A and *Q. Alba* medium grain A & 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 unlabelled 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 in triplicate by the method using 2:1 pentane : diethyl ether and also with diethyl ether and analysed by the method at three injector temperatures, 200°C, 225°C and 250°C, giving a total of six determinations for each of the three model wine solutions.

Investigation into acid catalysed **trans/cis** *isomerisation of oak lactone*

Solutions of oak lactone (*trans/cis* = 4:1, and *trans/cis* = 1:2, each approx. 10 mg total), obtained from column chromatography (as described in Chapter 2), were each made up in 12% aqueous ethanol (10 mL) acidified to pH = 1 with hydrochloric acid (32%, 10 M). The solutions were sealed in glass ampoules, and stored at 55°C for 53 days. The ampoules were then opened, and the contents analysed by GC-MS.

Investigations into the effect of pH and air on the quantitative analysis of vanillin and 4-ethylphenol in wine

A solution of $^{2}H_3$-vanillin (1.00 μg) and $^{2}H_4$-4-ethylphenol (2.00 μg) in ethanol (400 μL) was added to the wine (10.0 mL) in a test tube. Several experiments to determine the effect of wine pH on the analytical method were carried out. a) The internal standard was added and the wine was analysed. b) The pH of a red wine sample was adjusted to 6.0 with 2 M sodium hydroxide solution, with or without nitrogen blanketing of the headspace above the wine. The internal standard was then added and the sample analysed by the method. c) The internal standard was added to the red wine sample. The pH was then adjusted to 6.0 or 8.5 with 2 M sodium hydroxide solution without nitrogen blanketing of the headspace above the wine, and the sample analysed by the method. The wine was either at natural pH, or pH adjusted to 6.0 or 8.5 with 2 M sodium hydroxide. Diethyl ether (2-3 mL) was added and the mixture was shaken briefly. A portion of the ether layer was then placed in a vial ready for instrumental analysis. Extraction and GC/MS analysis of all wines was carried out in duplicate. The injection temperature was 220°C and the splitter was turned on after 45s. Other instrumental conditions were as described previously. These analyses were all done on the 5890II / 5971 HP GC/MS.

4.1.2 Vanillyl alcohol and vanillyl ethyl ether

Preparation of samples for analysis

For vanillyl alcohol quantitation, d_3-vanillyl alcohol (200 ng) in water (20 μL) was added to the wine (1 mL) and mixed thoroughly. The wine sample was passed through a phenyl solid phase extraction (SPE) cartridge (500 mg, Alltech, cat. no. 232300), (preconditioned by washing with methanol and water). Dichloromethane (3 mL) was passed through the column and the eluant concentrated to *ca* 1 mL (under a stream of nitrogen at room temp.) prior to analysis by GC/MS.

For quantitation of vanillyl ethyl ether, d_3-vanillyl ethyl ether (1.00 μg in 200 μL ethanol) was added to the wine (10 mL) in a test tube and mixed thoroughly. Diethyl ether (2-3 mL) was added and the mixture shaken briefly. A portion of the ethereal layer was placed in a vial for instrumental analysis.

Instrumental analyses

Samples were analysed with a Hewlett-Packard (HP) 5890A Series II gas chromatograph (GC) coupled to a HP 5971 mass spectrometer. The GC was fitted with an approx. 30 m x 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), linear velocity 31 cm/sec, flow rate was 0.72 mL/min, vacuum compensated at the mass spectrometer interface. The oven temperature was started at 60°C, held at this temperature for 2 min then increased to 250°C at 10°C/min and held at this temperature for 20 min. The injector was held at 220°C and the transfer line at 275°C. The sample volume injected was 3 μL. The splitter, at 30:1, was opened after 36 sec. The liner used was resilanised borosilicate glass, tapered at the injector end, with a plug (2-4 mm) of resilanised glass wool. 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* 35-450 for scan runs. For quantification of vanillyl alcohol and vanillyl

ethyl ether, mass spectra were recorded in the Selective Ion Monitoring (SIM) mode. The ions monitored in SIM runs were: *m/z* 140 and 185 for $^{2}H_3$-vanillyl ethyl ether (IS), m/*z* 137 and 182 for vanillyl ethyl ether, *m/z* 128, 140 and 157 for $^{2}H_3$-vanillyl alcohol (IS), m/*z* 125, 137 and 154 for vanillyl alcohol. The underlined ions were the ones used for quantitation (by peak area), with the other ions used to confirm the identity of the analyte. Selected fragment ions were monitored for 50 ms each.

Validation of the analytical method

Calibration curves for vanillyl alcohol and vanillyl ethyl ether were obtained by spiked standard additions to a dry white wine. Vanillyl alcohol was added to give concentrations of 0, 10, 20, 50, 100, 250, 500, 1000 and 2000 µg/L in the wine. Vanillyl ethyl ether was added to give concentrations of 0, 5, 10, 50, 100 and 150 µg/L in the wine. The calibration curves obtained were linear throughout the concentration range, with correlation coefficients (r^2) of 1.000 for vanillyl alcohol and 0.999 for vanillyl ethyl ether.

4.2 RESULTS AND DISCUSSION

4.2.1 Quantitative analysis of guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans* and *cis*-oak lactone and vanillin

The multi-component analysis for guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans* and *cis*-oak lactone and vanillin was validated by duplicate spiked standard additions to red, white and model wine matrices (details in materials and methods above). All of the curves obtained were linear throughout the concentration range. A typical calibration curve is shown in Figure 4.1 while Table 4.1 shows the correlations for all of the compounds in model, white and red wine matrices.

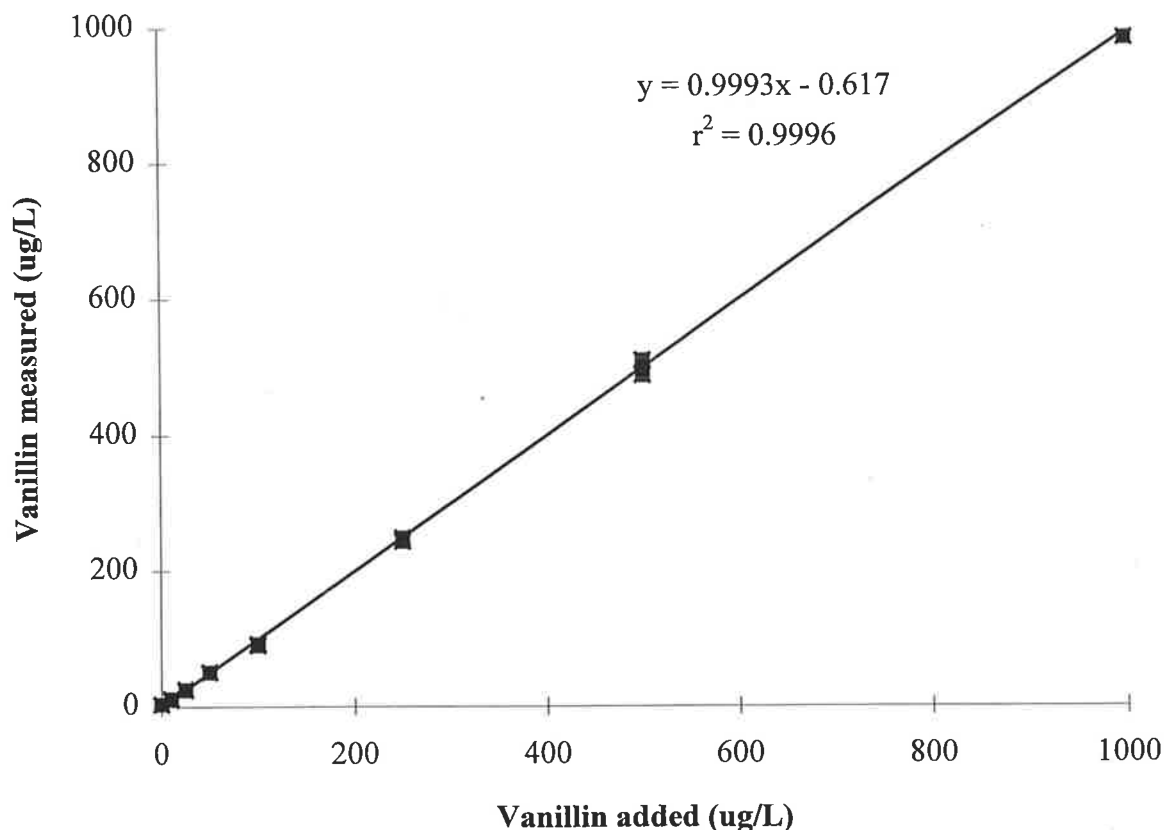


Figure 4.1 Standard addition curve for vanillin in red wine

Calibration curve obtained for vanillin analysis of duplicate spiked standard additions to a bag-in-box red wine. The points plotted are duplicate points for each concentration.

The curves for guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, and *trans* and *cis*-oak lactone are similar to the vanillin curve (Fig. 4.1) in red, white and model wines. The y-intercept will change depending on how much of the analyte of interest is present in the wine prior to spiking, eg. there was 4 μg/L vanillin in the bag-in-box red wine used in the vanillin curve. Over a period of months, the slope may change slightly due to spectral ratio drift, as discussed in section 4.2.1.3

Table 4.1 Correlation coefficients (r^2) determined over the indicated ranges of standard additions of compounds to red, white and model wine matrices

Compound	Red wine	White wine	Model wine	Range	n =
guaiacol	0.999	1.000		0–250 µg/L	8 x 2
guaiacol	0.998		1.000	0–1000 µg/L	10 x 2
4-methylguaiacol	1.000	1.000		0–250 µg/L	8 x 2
4-methylguaiacol	0.999		1.000	0–1000 µg/L	10 x 2
4-ethylphenol	0.999	0.999	1.000	0–1000 µg/L	10 x 2
4-ethylphenol	0.993	0.996	0.996	0–5000 µg/L	10 x 2
4-ethylguaiacol	1.000	0.999	0.999	0–1000 µg/L	10 x 2
4-ethylguaiacol	0.996	0.996	0.994	0–5000 µg/L	10 x 2
trans-oak lactone	1.000		1.000	0–1000 µg/L	10 x 2
cis-oak lactone	1.000		1.000	0–1000 µg/L	10 x 2
vanillin	1.000		0.999	0–1000 µg/L	10 x 2

The data shown in the above table is for injections at 200°C. The same pentane : ether (2:1) extracts were also run at 220°C and 250°C giving similar results. Thus, there were no injector block artefact generation effects observed for these wine extracts. This is not the case, however, for all extracts of oak shavings as explained in detail later in this section.

Table 4.2 Repeatability of the oak volatiles assay at higher concentrations

Amounts measured in μg/L – septuplicate determinations

Model wine (10% ethanol in water, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid)

	spike level	mean	st dev	st dev / mean
guaiacol	200	204	3	1.6%
4-methylguaiacol	200	194	10	5.4%
4-ethylphenol	500	513	12	2.3%
4-ethylguaiacol	500	518	18	3.4%
***trans*-oak lactone**	500	499	15	2.9%
***cis*-oak lactone**	500	505	14	2.8%
vanillin	500	519	19	3.6%

Oaked white wine (1996 Semillon)

	spike level	mean	st dev	st dev / mean
guaiacol	200	212	6	2.8%
4-methylguaiacol	200	203	6	2.9%
4-ethylphenol	500	518	13	2.4%
4-ethylguaiacol	500	512	23	4.5%
***trans*-oak lactone**	500	532	14	2.6%
***cis*-oak lactone**	500	500	16	3.2%
vanillin	500	627	36	5.7%

Oaked red wine (1995 Cabernet Sauvignon / Shiraz / Malbec)

	spike level	mean	st dev	st dev / mean
guaiacol	200	229	6	2.5%
4-methylguaiacol	200	201	8	3.8%
4-ethylphenol	500	708	19	2.7%
4-ethylguaiacol	500	544	35	6.5%
***trans*-oak lactone**	500	571	13	2.3%
***cis*-oak lactone**	500	606	31	5.0%
vanillin	500	605	12	2.0%

Model, white and red wines were spiked and analysed in septuplicate to show the repeatability of the extraction and analysis. Extracts were all run on the HP 5890 II / 5971 GC/MS.
st dev = standard deviation, st dev / mean = coefficient of variance = relative standard deviation
The red and white wines give higher values than that added because they contained unlabelled analytes of interest prior to spiking (unsurprising considering the wines were made in contact with oak).

Table 4.3 Repeatability of the oak volatiles assay at lower concentrations

Amounts measured in μg/L – septuplicate determinations

Model wine (10% ethanol in water, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid)

	spike level	mean	st dev	st dev / mean
guaiacol	10	9.7	0.1	1.3%
4-methylguaiacol	10	9.7	0.2	1.7%
4-ethylphenol	25	25	0.5	2.0%
4-ethylguaiacol	25	23	0.4	1.7%
***trans*-oak lactone**	25	25	0.4	1.8%
***cis*-oak lactone**	25	25	0.5	2.0%
vanillin	25	25	1.1	4.3%

Bag-in-box dry white wine

	spike level	mean	st dev	st dev / mean
guaiacol	10	10.6	0.1	1.3%
4-methylguaiacol	10	9.9	0.1	1.3%
4-ethylphenol	25	26	0.5	2.1%
4-ethylguaiacol	25	27	0.4	1.4%
***trans*-oak lactone**	25	26	0.5	1.9%
***cis*-oak lactone**	25	29	0.7	2.4%
vanillin	25	35	0.9	2.6%

Bag-in-box red wine (Claret)

	spike level	mean	st dev	st dev / mean
guaiacol	10	14.8	0.5	3.2%
4-methylguaiacol	10	10.7	0.2	1.9%
4-ethylphenol	25	29	0.6	2.0%
4-ethylguaiacol	25	27	0.5	2.0%
***trans*-oak lactone**	25	28	0.6	2.1%
***cis*-oak lactone**	25	47	0.5	1.0%
vanillin	25	38	1.0	2.6%

Model, white and red wines were spiked and analysed in septuplicate to show the repeatability of the extraction and analysis. Extracts were all run on the HP 5890 II / 5971 GC/MS.
st dev = standard deviation, st dev / mean = coefficient of variance = relative standard deviation
The red and white wines give higher values than that added because they contained unlabelled analytes of interest prior to spiking, indicating that some contact with oak (maybe as oak chips) had occurred during winemaking.

In comparison to the techniques in the literature (as discussed at the end of this section), analysis of the oak volatiles guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *cis*- and *trans*-oak lactone and vanillin, by stable isotope dilution (except for 4-ethylguaiacol) coupled with GC/MS using selected ion monitoring was precise, accurate, robust and rapid (sample preparation time was reduced from several days in the case of eg. Sefton *et al.* 1993b) to 2-3 minutes, and required only a small sample of wine (5 mL), and obtained detection at 1 μg/L and often lower. Also all seven of these compounds could be accurately assayed in a single analysis, even though they were not all of similar chemical structures. As can be seen from figure 4.1 and tables 4.1 – 4.3, the analytical method is precise and accurate for all the compounds in all the matrices analysed.

The solvent system of pentane-diethyl ether (2:1) gave the best signal to noise ratio overall for all compounds; however, guaiacol and 4-methylguaiacol were best with pentane only and vanillin analysis was two to three times more sensitive with ether only. Similar sensitivity was obtained for SPME and pentane extractions for all compounds except vanillin, which was less soluble in pentane and was either not volatile enough to obtain a good concentration in the headspace, or had little affinity for both polydimethylsiloxane (PDMS) and Carbowax / divinylbenzene SPME fibres.

4.2.1.1 Avoidance of co-eluting peaks during gas chromatography

For oak lactone, the greatest sensitivity was obtained by monitoring the base peak fragments of the labelled and unlabelled compounds, *m/z* 101 and 99 respectively. These odd numbered ions are, however, common to the spectra of a number of wine and wood components, and caution is required if they are to be used in quantification, especially *m/z* 101. Although smaller in comparison to the base peak, the even-number ion *m/z* 90 of the internal standards proved more reliable than *m/z* 101 for quantification. The relative ratios of ions 118, 132 and 160 (M^+) in the internal standard, and 114, 128 and 156 (M^+) in the analyte were also monitored to ensure peak homogeneity. Similar

co-elution problems occasionally occurred for vanillin, although these were rarer. The molecular ions for both vanillin (*m/z* 152) and d_3-vanillin (*m/z* 155) usually had the best signal to noise ratio, but occasionally the M-1 fragments of *m/z* 151 and / or *m/z* 154, respectively, were used to avoid interferences from co-eluting peaks. In these cases there was little sensitivity lost as the M-1 fragments for vanillin and d_3-vanillin were of approximately equal intensity to that of the corresponding molecular ion, which is unsurprising since vanillin is an aldehyde.

4.2.1.2 Stability of standards

In general, all the deuterated standards were acid stable. The stability of the deuterium labels at wine pH can be assumed, as no loss of deuterium was ever observed under the conditions of the analysis, for any of the compounds analysed. This was determined by scrutiny of the ion ratios for every compound analysed in every analysis. Further experiments were designed for the compounds of most interest.

Tests were done on d_4-4-ethylphenol (Appendix II) as the ortho deuterium atoms could be exchanged under strong acidic conditions as shown in Scheme 2.5. No exchange of the deuterium atoms took place during sample preparation or chromatography.

An investigation into the possible acid catalysed *cis / trans* isomerisation of oak lactone was conducted. Two model wine samples of the oak lactones, enriched in the *cis*- and *trans*- isomers respectively, were heated to 55°C at pH 1 for 53 days. No change in the isomer ratios was observed during this time and no other products were formed. Significant acid-catalysed *cis / trans* isomerization does not, therefore, take place in the weakly acidic medium of wines and spirits during preparation of samples for analysis. Indeed, such acid catalysed isomerisation will also not take place during normal storage, even over prolonged periods, as has been suggested elsewhere (Chatonnet 1991, Waterhouse and Towey 1994, Piggott *et al.* 1995).

4.2.1.3 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 (eg. d_3-guaiacol vs unlabelled

guaiacol, d_4-4-ethylphenol vs unlabelled 4-ethylphenol and 4-ethylguaiacol, d_4-*cis*-oak lactone vs unlabelled *cis*-oak lactone) varied slightly (+/- 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 were analysed, replicate reference standards were made containing known concentrations of each deuterium labelled IS and each unlabelled analyte. Also, with each batch, a reference standard was run which contained just the isotopically labelled standards portion, to account for ions common to both labelled and unlabelled compounds (eg normal oak-lactone contains a small (< 2%) ion at *m/z* 97, which corresponds to a *m/z* 99 in the d_4-oak lactone – this needs to be accounted for, or else measured unlabelled oak lactone concentration would be falsely inflated by the contribution from the labelled standard). 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 unlabelled compounds used to make up the reference mix.

4.2.1.4 SPME analysis of the oak volatiles

Guaiacol, 4-methylguaiacol, 4-ethylphenol and *trans*- and *cis*-oak lactone in wine could be determined with equal facility by solid-phase micro-extraction (SPME) of the headspace above the wine. In practice, this technique gave cleaner chromatograms, but with lower sensitivity than those using the organic solvent extracts. Nevertheless, detection limits of 1 µg/L were usually still obtained. 4-Ethylguaiacol analyses by SPME were not as accurate or repeatable as the other analytes because it was quantitated versus d_4-4-ethylphenol rather than an exact isotopic analogue (eg. d_3-4-ethylguaiacol). As the volatility of d_4-4-ethylphenol and 4-ethylguaiacol are not identical, differences in temperature, extraction time, salinity, ionic strength, affinity for the SPME fibre, etc. had more effect on the ratio of 4-ethylguaiacol to d_4-4-ethylphenol

than eg. 4-ethylphenol to d_4-4-ethylphenol.

Special attention was given to oak lactone and vanillin because they have relatively low volatilities compared with the other analytes. Optimised conditions gave good results for oak lactone but the level of quantitation for vanillin was seldom better than 100 μg/L. The optimisation study for oak lactone showed that the use of PDMS (100 μm Polydimethylsiloxane), as the bonded phase, proved to be a more efficient adsorbent of the lactones than was 65 μm Carbowax-divinylbenzene (DVB), partially crosslinked. The PDMS fibre also gave more efficient desorption in the gas chromatograph injector, because it was stable at 270°C whereas the Carbowax-DVB fibre used was stable only to 220°C. PDMS extracted an extra 10% *trans*-oak lactone (and d_4-*trans* oak lactone) relative to *cis*-oak lactone (and d_4-*cis* oak lactone) when compared to Carbowax-DVB. However, the Carbowax-DVB fibre was, in general, preferred for the analysis of oak lactones as the chromatograms were cleaner (ie. better signal to noise) than those obtained with the PDMS fibre when real wine and oak extract samples were analysed.

The optimisation studies were all done on 5 mL aliquots of the same red wine (an unwooded Coonawarra Cabernet Sauvignon) by the same SPME methodology as that shown in the analytical method (section 4.1.1) except that the nature of the fibre (PDMS or Carbowax-DVB), salt addition (1 g or 0 g), or stirring time (0, 10, 20, 30 & 60 min) were varied. All determinations were done in duplicate.

Salting the wine extracts prior to SPME gave recoveries 2.5 times better than unsalted wine. The more volatile *trans* isomer was salted out 1.14 times more readily than the *cis* isomer. Stirring the wine extracts during SPME gave approximately 10% higher recoveries for extractions up to 45 min. duration, but made no detectable difference after 60 min.

Efficiency of adsorption by the SPME fibre appeared to be controlled mainly by kinetic factors, especially for the less volatile *cis*-isomer, as demonstrated by the time study shown in Fig. 4.2. Long adsorption times gave greater sensitivity.

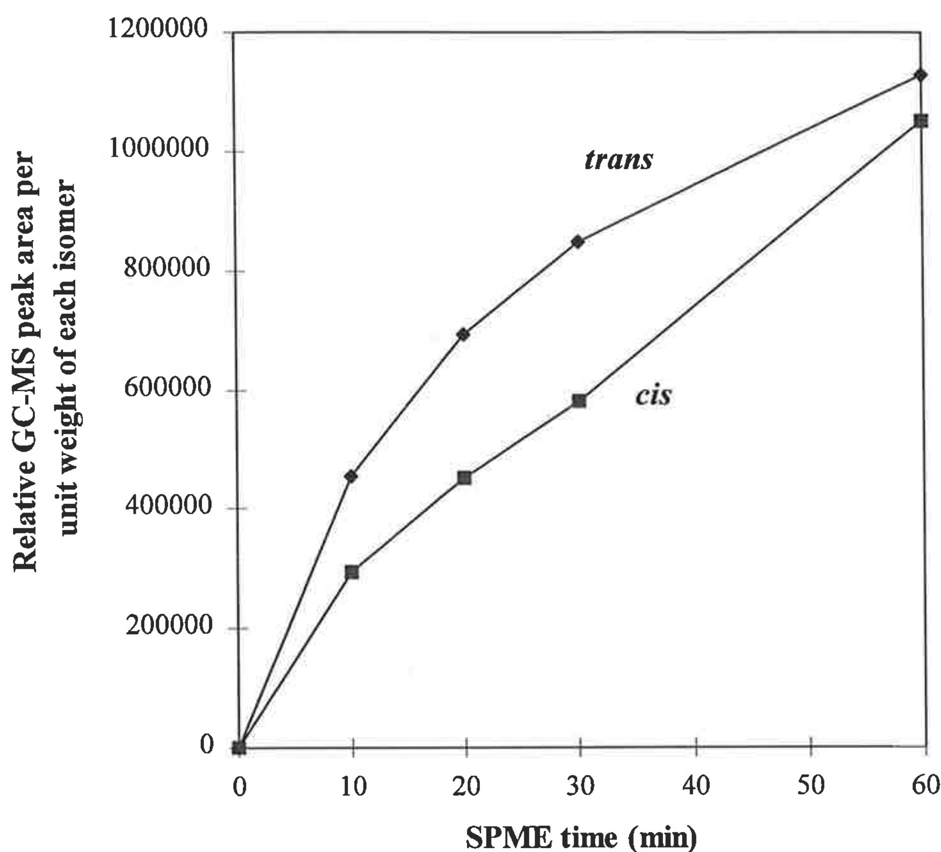


Figure 4.2 Solid phase microextraction (SPME) of oak lactone isomers from model wine over time

Model wine was 10% ethanol in water, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid. Each point shown is the mean of two determinations. This time plot was done under optimised conditions (5 mL wine +IS mix, Carbowax / DVB fibre, 1 g salt, room temperature).

In practice, an absorption time of 20 min. allowed for a level of detection below 1 μg/L and accuracy of the analysis was not compromised as the internal standards were absorbed at the same rate as their non-isotopically labelled analogues.

The standard curve for the optimum conditions is shown in Figure 4.3.

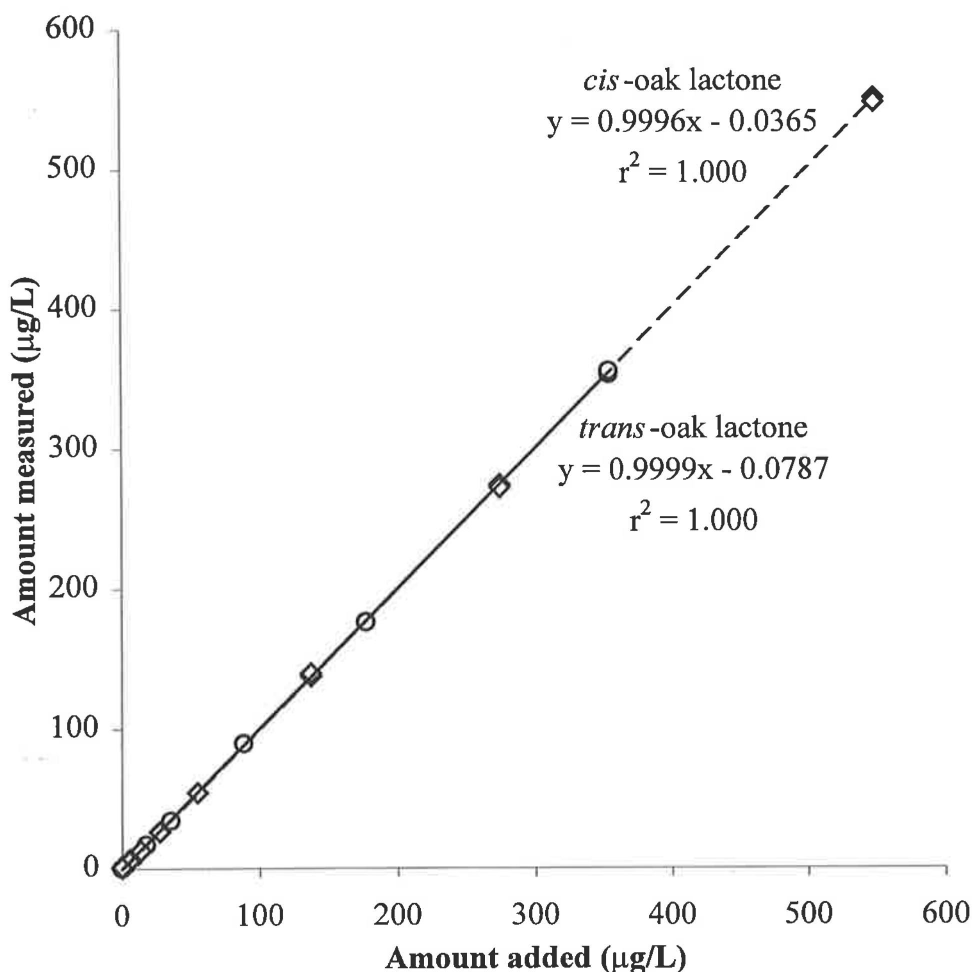


Figure 4.3 SPME standard addition curves for *cis*- and *trans*-oak lactone

The standard curves plotted above were obtained by spiked duplicate additions (0 and 10-1000 μg/L, N=8 x 2) of commercial synthetic oak lactone (Interchim., 90.4% pure by GC/MS, *cis/trans* = 1.55/1.00 by GC/MS) to a bottled red wine, which was extracted by the optimised SPME method, giving curves linear throughout the concentration range: r^2 = 1.000 (0, and 5.50 to 550 μg/L added *cis*-oak lactone, N=8 x 2, y=0.9996x – 0.0365) [dotted line in the plot, a diamond represents each point]; r^2 = 1.000 (0, and 3.54 to 354 μg/L added *trans*-oak lactone, N=8 x 2, y=0.9999x – 0.0787) [solid line in the plot, a circle represents each point].

4.2.1.5 Studies on the artefactual generation of oak volatiles during the analysis of oak extracts – injector and solvent effects

To test the possibility that oak volatiles were released from bound forms by thermal degradation in the gas chromatograph injector block during the analysis, concentrated model wine extracts of oak shavings were analysed under a variety of conditions. Fine shavings were taken from six oak wood samples, two staves of *Quercus alba*-fine grain, two of *Quercus alba*-medium grain, and two of Chestnut Oak (*Q prinus*). Half of the shavings from each oak sample were heated, the other half were left unheated, prior to soaking all twelve samples of shavings in model wine at room temperature. Preliminary experiments (data not shown) showed six of these oak staves showed potential for artefactual generation effects. Thus separate 5 mL aliquots of these six (*Q. Alba* fine grain B and Chestnut Oak A & B, heated and unheated) were extracted and analysed in triplicate by SPME and also by liquid-liquid extraction with pentane, pentane : diethyl ether (2:1) and diethyl ether and each replicate was analysed by the method at three different injector block temperatures; 200°C, 225°C and 250°C, giving a total of thirty determinations for each model wine solution. Separate 5 mL aliquots from the remaining six solutions were extracted and analysed in triplicate by SPME and also by liquid-liquid extraction with pentane : diethyl ether (2:1) and injected at two different injector block temperatures; 200°C and 250°C, giving a total of nine determinations for each model wine solution of *Q. Alba* fine grain A and *Q. Alba* medium grain A & B, heated and unheated.

To be thorough, and to show that any artefact formation observed is due to oak derived precursors, at the same time the extractions and analyses of the oak extracts above were performed, model wine (with no contact with oak shavings) was spiked in triplicate with the unlabelled oak compounds of interest and extracted in triplicate with diethyl ether and also with pentane : diethyl ether (2:1) and analysed by the method at three injector temperatures, 200°C, 225°C and 250°C, giving a total of six determinations for each of the three model wine solutions. (Data shown in table 4.4 below). There was good reproducibility for all analytes and good accuracy for all of them, except 4-ethylguaiacol. In the case of this last compound, it did not have an exact isotopic

analogue as internal standard but used d_4-4-ethylphenol, which gave acceptable accuracy for pentane : diethyl ether (2:1) extracts injected at 200°C, but was less accurate at higher injector temperatures and least accurate for ether extracts. It is unsurprising that 4-ethylguaiacol is less soluble in the ether, the more polar solvent used for the liquid-liquid extraction, than is 4-ethylphenol. The injector temperature differences are due to the different volatility of 4-ethylphenol and 4-ethylguaiacol.

If the same concentration of an oak volatile is measured, independent of whether the wine is extracted by SPME or liquid / liquid extraction at each injector block temperature, then it can be concluded that there was no artefactual formation of the compound of interest from precursors as a result of the analysis. This was the case for the spiked model wine controls (with no oak wood extraction) and for some of the real oak extracts (eg Figures 4.2 and 4.3) but not all (as evident from tables 4.5 to 4.8 and Figures 4.4 to 4.7).

Table 4.4 Spiked model wines analysed at the same time as the oak extracts

Concentration measured in μg/L (relative standard deviation %)

	pentane : diethyl ether (2:1) extraction		
	Inj. 200°C	**Inj. 225°C**	**Inj. 250°C**
guaiacol	202 (0.9%)	205 (1.0%)	203 (1.5%)
4-methylguaiacol	198 (1.8%)	201 (2.1%)	201 (1.4%)
4-ethylphenol	507 (1.7%)	502 (1.0%)	503 (1.5%)
4-ethylguaiacol	480 (1.0%)	453 (0.8%)	467 (1.0%)
***trans*-oak lactone**	506 (1.9%)	507 (1.4%)	508 (3.3%)
***cis*-oak lactone**	516 (1.5%)	514 (1.5%)	520 (2.6%)
vanillin	497 (0.7%)	500 (1.0%)	506 (2.9%)

	diethyl ether extraction		
	Inj. 200°C	**Inj. 225°C**	**Inj. 250°C**
guaiacol	196 (0.9%)	196 (1.7%)	210 (3.3%)
4-methylguaiacol	200 (1.3%)	199 (1.5%)	200 (1.5%)
4-ethylphenol	494 (1.4%)	489 (1.7%)	491 (1.7%)
4-ethylguaiacol	389 (8.3%)	356 (7.9%)	366 (10.2%)
***trans*-oak lactone**	503 (0.7%)	503 (1.0%)	503 (1.3%)
***cis*-oak lactone**	500 (1.0%)	501 (1.2%)	505 (1.5%)
vanillin	500 (0.9%)	501 (1.5%)	510 (2.9%)

Model wine used was 10% ethanol / water, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid, spiked with 200 μg/L guaiacol and 4-methylguaiacol and 500 μg/L 4-ethylphenol, 4-ethylguaiacol, *trans*-oak lactone, *cis*-oak lactone and vanillin. All determinations were done in triplicate. Inj. means injection temperature.

Table 4.5 Summary - significant effects on guaiacol concentration of liquid injection at 200°C vs SPME

Oak Sample	Solvent System	L/L : SPME	Significance
Q. Alba Fine Grain B - Raw	2:1 P:E	115%	***
Q. Alba Fine Grain B - Raw	Ether	144%	***
Chestnut Oak A - Raw	2:1 P:E	104%	***
Chestnut Oak A - Raw	Ether	107%	***
Chestnut Oak B - Raw	2:1 P:E	107%	***
Chestnut Oak B - Raw	Ether	113%	***
Q. Alba Fine Grain B - Heated	Ether	139%	***
Q. Alba Med. Grain A - Heated	*2:1 P:E*	*110%*	***
Q. Alba Med. Grain B - Heated	*2:1 P:E*	*117%*	***
Chestnut Oak A - Heated	2:1 P:E	104%	**
Chestnut Oak A - Heated	Ether	221%	***
Chestnut Oak B - Heated	Pentane	130%	***
Chestnut Oak B - Heated	Ether	178%	***

Guaiacol was the only analyte that gave significant artefactual formation at 200°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.

Italics means that the oak wood extract was only analysed by 2:1 pentane : ether (2:1 P:E) liquid-liquid extraction and SPME.

** = 0.01 significance, 1% level, 99% significant
*** = 0.001 significance, 0.1% level, 99.9% significant

Full data is given in Appendix I.

Table 4.6 Summary - significant effects of liquid injection at 225°C vs SPME

Oak Sample	Solvent System	Analyte	L/L : SPME	Significance
Q. Alba Fine Grain B - Raw	2:1 P:E	Guaiacol	116%	***
Q. Alba Fine Grain B - Raw	Ether	Guaiacol	287%	***
Q. Alba Fine Grain B - Raw	Ether	4-MeG	103%	***
Q. Alba Fine Grain B - Raw	Ether	cis-OL	106%	***
Chestnut Oak A - Raw	2:1 P:E	Guaiacol	109%	***
Chestnut Oak A - Raw	Ether	Guaiacol	135%	***
Chestnut Oak A - Raw	Ether	4-MeG	150%	***
Chestnut Oak B - Raw	2:1 P:E	Guaiacol	107%	*
Chestnut Oak B - Raw	Ether	Guaiacol	147%	***
Chestnut Oak B - Raw	Ether	4-MeG	136%	***
Q. Alba Fine Grain B - Heated	Ether	Guaiacol	315%	***
Chestnut Oak A - Heated	2:1 P:E	Guaiacol	122%	*
Chestnut Oak A - Heated	Ether	Guaiacol	467%	***
Chestnut Oak A - Heated	Ether	4-MeG	103%	***
Chestnut Oak B - Heated	Pentane	Guaiacol	164%	**
Chestnut Oak B - Heated	2:1 P:E	Guaiacol	113%	**
Chestnut Oak B - Heated	Ether	Guaiacol	321%	***

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.

* = 0.05 significance, 5% level, 95% significant
** = 0.01 significance, 1% level, 99% significant
*** = 0.001 significance, 0.1% level, 99.9% significant

2:1 P:E means 2:1 pentane : ether
4-MeG means 4-methylguaiacol
cis-OL means *cis*-oak lactone

Full data is given in Appendix I.

Table 4.7 Summary - significant effects of liquid injection at 250°C vs SPME

Oak Sample	Solvent System	Analyte	L/L : SPME	Significance
Q. Alba Fine Grain A - Raw	*2:1 P:E*	*Guaiacol*	*242%*	***
Q. Alba Fine Grain B - Raw	2:1 P:E	Guaiacol	128%	***
Q. Alba Fine Grain B - Raw	2:1 P:E	cis-OL	107%	***
Q. Alba Fine Grain B - Raw	Ether	Guaiacol	768%	***
Q. Alba Fine Grain B - Raw	Ether	4-MeG	106%	***
Q. Alba Fine Grain B - Raw	Ether	cis-OL	112%	***
Q. Alba Med. Grain A - Raw	*2:1 P:E*	*Guaiacol*	*271%*	*
Q. Alba Med. Grain B - Raw	*2:1 P:E*	*Guaiacol*	*193%*	*
Chestnut Oak A - Raw	2:1 P:E	Guaiacol	115%	**
Chestnut Oak A - Raw	Ether	Guaiacol	227%	***
Chestnut Oak A - Raw	Ether	4-MeG	188%	***
Chestnut Oak A - Raw	Ether	cis-OL	103%	***
Chestnut Oak B - Raw	2:1 P:E	Guaiacol	110%	***
Chestnut Oak B - Raw	Ether	Guaiacol	234%	***
Chestnut Oak B - Raw	Ether	4-MeG	164%	***
Chestnut Oak B - Raw	Ether	cis-OL	103%	***
Q. Alba Fine Grain A - Heated	*2:1 P:E*	*Guaiacol*	*155%*	***
Q. Alba Fine Grain B - Heated	Pentane	Guaiacol	108%	***
Q. Alba Fine Grain B - Heated	Ether	Guaiacol	750%	***
Q. Alba Med. Grain A - Heated	*2:1 P:E*	*Guaiacol*	*128%*	***
Q. Alba Med. Grain B - Heated	*2:1 P:E*	*Guaiacol*	*126%*	***
Chestnut Oak A - Heated	2:1 P:E	Guaiacol	145%	***
Chestnut Oak A - Heated	Ether	Guaiacol	1110%	***
Chestnut Oak A - Heated	Ether	4-MeG	104%	***
Chestnut Oak A - Heated	Ether	cis-OL	104%	***
Chestnut Oak B - Heated	Pentane	Guaiacol	183%	*
Chestnut Oak B - Heated	2:1 P:E	Guaiacol	137%	**
Chestnut Oak B - Heated	Ether	Guaiacol	818%	***
Chestnut Oak B - Heated	Ether	4-MeG	106%	**

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.

4-MeG = 4-methylguaiacol, cis-OL = *cis*-oak lactone

Italics means that the oak wood extract was only analysed by 2:1 pentane : ether (2:1 P:E) and SPME.

* = 0.05 significance, 5% level, 95% significant
** = 0.01 significance, 1% level, 99% significant
*** = 0.001 significance, 0.1% level, 99.9% significant

Table 4.8 Summary - significant effects of liquid injection at 225°C and 250°C vs 200°C on the measured concentration of vanillin in oak extracts

Oak Sample	Solvent System	Injection Temp. (°C)	Ratio vs Inj 200°C	Significance
Q. Alba Med. Grain B - Raw	*2:1 P:E*	*250*	*107%*	**
Chestnut Oak B - Raw	2:1 P:E	225	104%	***
Chestnut Oak B - Raw	2:1 P:E	250	104%	***

Italics means that the oak wood extract was only analysed by 2:1 pentane : ether (2:1 P:E) liquid-liquid extraction and SPME.

"Ratio vs Inj 200°C" denotes the ratio of the amount of vanillin measured by liquid-liquid extraction at the injection temperature shown in the middle column over the amount of vanillin measured for the same extract injected at 200°C, expressed as a percentage. As discussed in the text, SPME gave poor levels of detection for vanillin, and thus were not very accurate for vanillin quantitation due to signal to noise interference. Liquid – liquid extraction with pentane also gave low sensitivity for vanillin, but was better than SPME analyses of vanillin.

** = 0.01 significance, 1% level, 99% significant

*** = 0.001 significance, 0.1% level, 99.9% significant

Full data is given in Appendix I.

It is of interest that the effect of oak volatile formation varies according to the analyte.

Guaiacol is the volatile that shows the largest effects, with increases of over ten times as much guaiacol possible, especially when higher injector temperature (250°C) and more polar solvent systems such as ether are involved. Indeed guaiacol is the only volatile to show significant artefact formation at an injector temperature of 200°C, or when pentane is used as the sole extracting solvent. In general, there was much more guaiacol formed in the heated wood as compared to the unheated, which is in agreement with the results in Chapter 7 and the literature in general.

4-Methylguaiacol usually had low 0-6% (but statistically significant) artefact formation when ether was the extracting solvent at higher injector temperatures. Raw chestnut oak gave the biggest 4-methylguaiacol increases of 1.64 to 1.88 times as much.

Although the artefactual formation of *cis*-oak lactone is often extremely significant statistically, the total increase is only 0-12%, highest when more polar solvents (ether), higher injector temperature (250°C) and unheated wood are factors.

No 4-ethylphenol and very little 4-ethylguaiacol were formed by heating the oak; hence, it is unsurprising that there is no significant artefact formation for these compounds.

The sometimes major differences in the amount of artefactually generated volatiles (eg. guaiacol) measured could be due to variability in the residence time of the needle in the GC injector block (approx. 0.1 second) and another source of variation is the rates of solubility of the precursors as compared to the isotopically labelled standard (which is equally soluble to the analyte of interest, but not its precursors.) If precise use of an isotopically labelled standard, strict adherence to good analytical technique, and thorough and critical checking of results were not done, these anomalies would not have been noticed and been simply glossed over as analytical error.

4.2.2 Robustness of the analytical method

This section illustrates several points including accuracy, reproducibility, artefact formation and peak homogeneity. To follow are selected graphical representations of the data obtained in the previous section that portray both the strengths and pitfalls of the method developed in this thesis to measure oak volatiles.

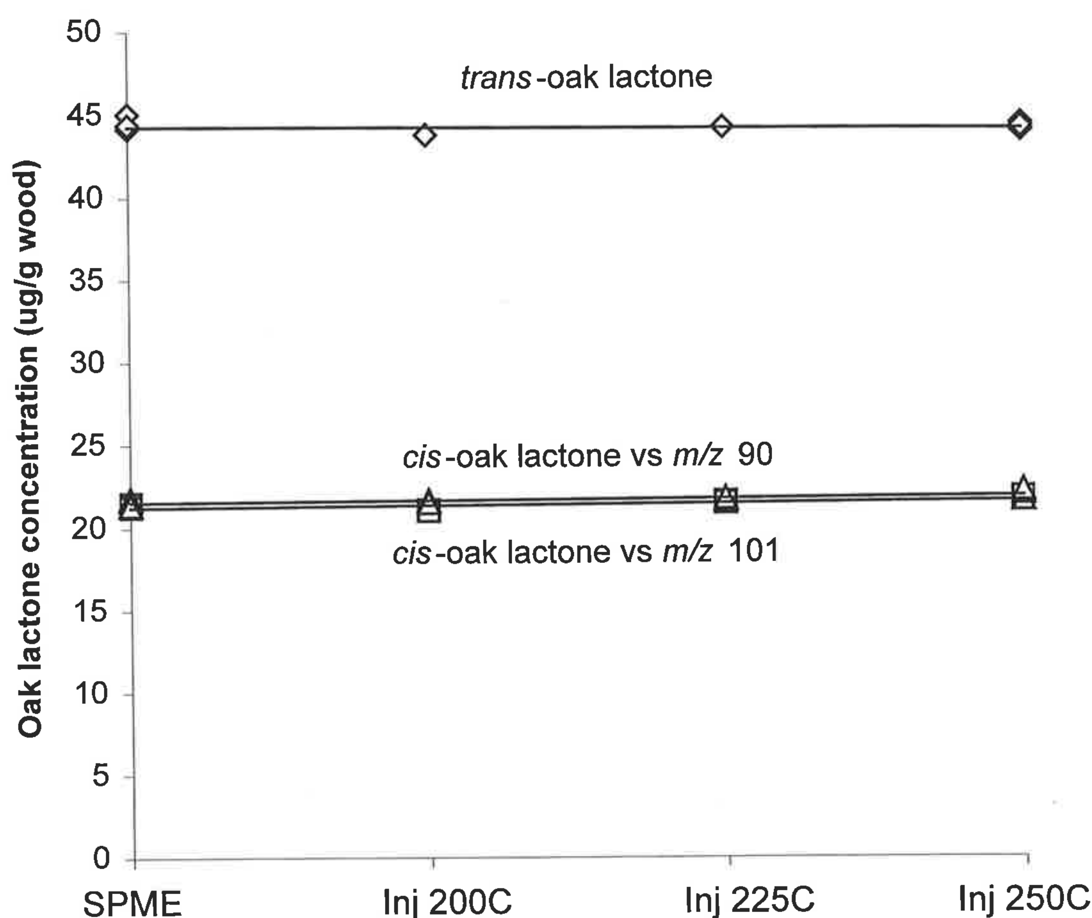


Figure 4.2 Oak lactone concentration measured in heated Chestnut Oak A as affected by injection temperature

This figure shows the concentrations measured by SPME and by liquid-liquid extraction with 2:1 pentane : ether at three injector temperatures. All determinations were done in triplicate.

Figure 4.2 is a good example of no artefact formation in the analysis of oak lactone by SPME or 2:1 pentane : ether extraction due to increase in injector block temperature. The same concentration of both oak lactone isomers is observed regardless of the injection block temperature or whether the extraction was done via 2:1 pentane : ether or SPME. There was no co-eluter with the *m/z* 101 ion of d_4-*cis* oak lactone as evident from the approximately equal concentrations of *cis*-oak lactone whether quantitated vs the *m/z* 90 or *m/z* 101 ions of d_4-*cis* oak lactone.

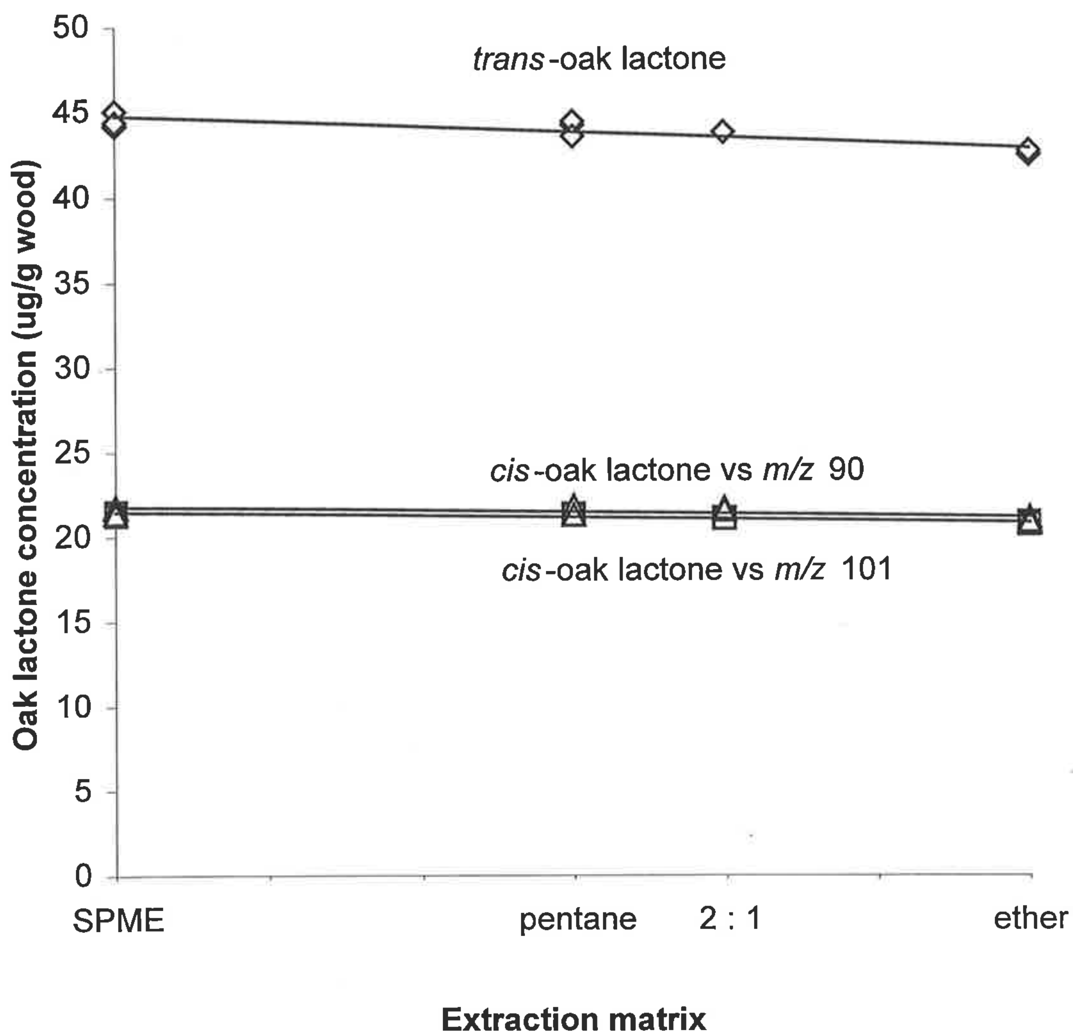


Figure 4.3 Oak lactone concentration measured in heated Chestnut Oak A – SPME and liquid/liquid extraction with pentane, 2:1 pentane : ether and ether (all liquid injections at 200°C)

All determinations were done in triplicate.

For this oak sample, with extraction by either pentane, pentane : ether (2:1) or ether, no artefacts were found at a GC injector block temperature of 200°C. This may be because these extracts contain very small amounts of precursors or this temperature is too low to break down the precursors.

There is no significant difference between the concentration of *cis*-oak lactone calculated versus either the *m/z* 101 or *m/z* 90 ion of d_4-*cis* oak lactone, therefore there are also no co-eluters with the *m/z* 101 ion.

If there are no other interferences the concentration calculated versus *m/z* 90 will always be slightly higher than that calculated versus *m/z* 101 due to the threshold effect since the relative intensity of *m/z* 90 is approx. 20% that of *m/z* 101 in d_4-*cis* oak lactone. This small effect can be seen in Figure 4.3.

Another small effect that can be seen in Figure 4.3, especially for *trans*-oak lactone, is that the relative concentrations measured by the different extraction methods are SPME > pentane > pentane : ether (2:1) > ether. These differences are small, but the concentration measured by SPME is 4.5% greater than that measured by ether in Figure 4.3. Similar small effects are observed for some analytes in other oak extracts in Appendix I and Chapter 7.2.3. Spiked model wine extracts, containing no oak extraction material at all, still showed this effect (data not shown). Thus this effect is not due to co-eluting compounds or soluble precursors and hence may possibly be due to small differences in the affinity of the SPME fibre for the slightly more lipophilic deuterium rather than hydrogen protons. This effect is so small that the GC retention times of the labelled and unlabelled molecules are identical to within 0.02 min, but always the deuterated analogue elutes first. It is a measure of the precision of the analysis that this effect can be seen at all.

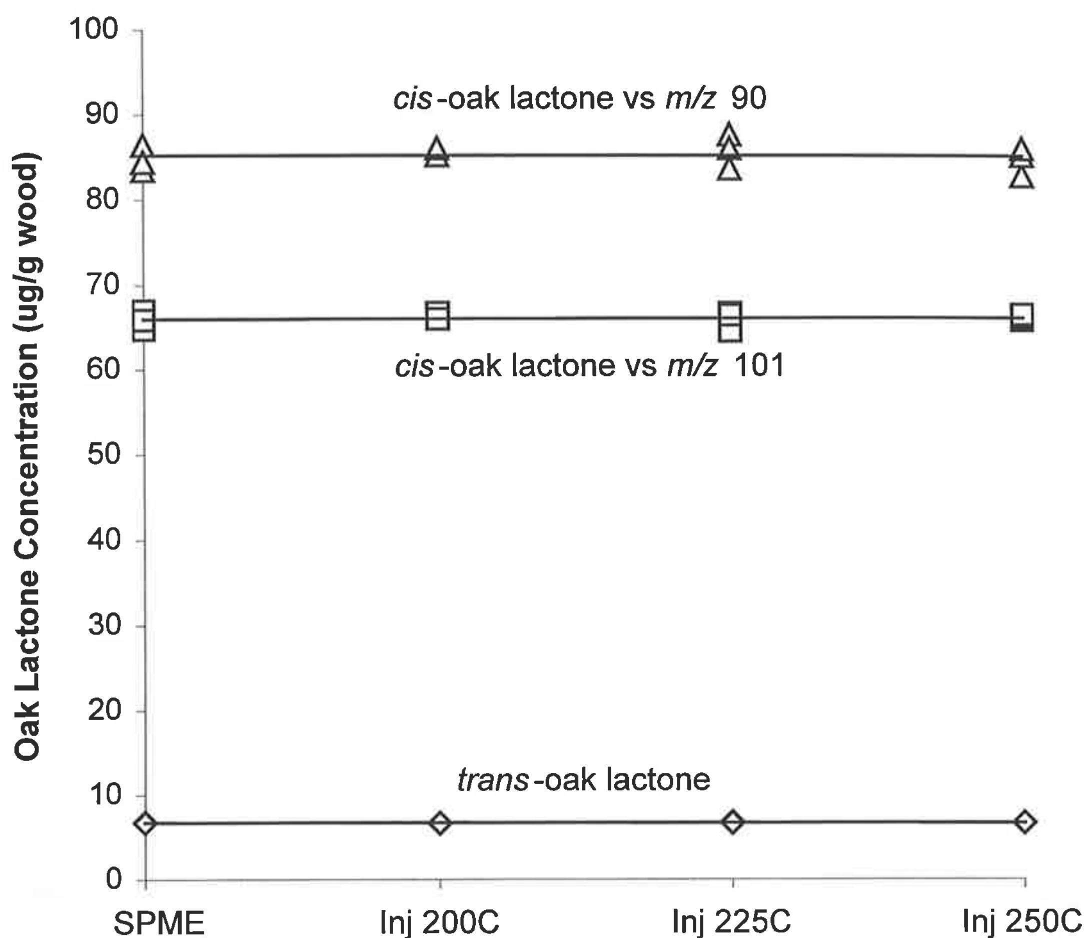


Figure 4.4 Oak lactone concentration measured in unheated *Q. Alba* Fine Grain B – SPME and pentane extraction injected at different temperatures

All determinations were done in triplicate.

In this figure there are no artefacts resulting from increased injector temperature, however a co-eluter with the *m/z* 101 ion of d_4-*cis*-oak lactone precisely and systematically gives the wrong quantitation for oak lactone, over 20% lower than the correct quantitation obtained versus the *m/z* 90 ion of d_4-*cis*-oak lactone. This co-elution effect was not visible from peak shape, as the peak was symmetrical. The error was observed by comparing the relative response ratios of the other qualifier ions, eg *m/z* 90 versus *m/z* 118, 132 and 160 were correct whereas *m/z* 101 versus these same three ions was out by approximately 20% in each case. Variations in the GC column temperature program confirmed that a co-eluting peak with *m/z* 101 was the problem.

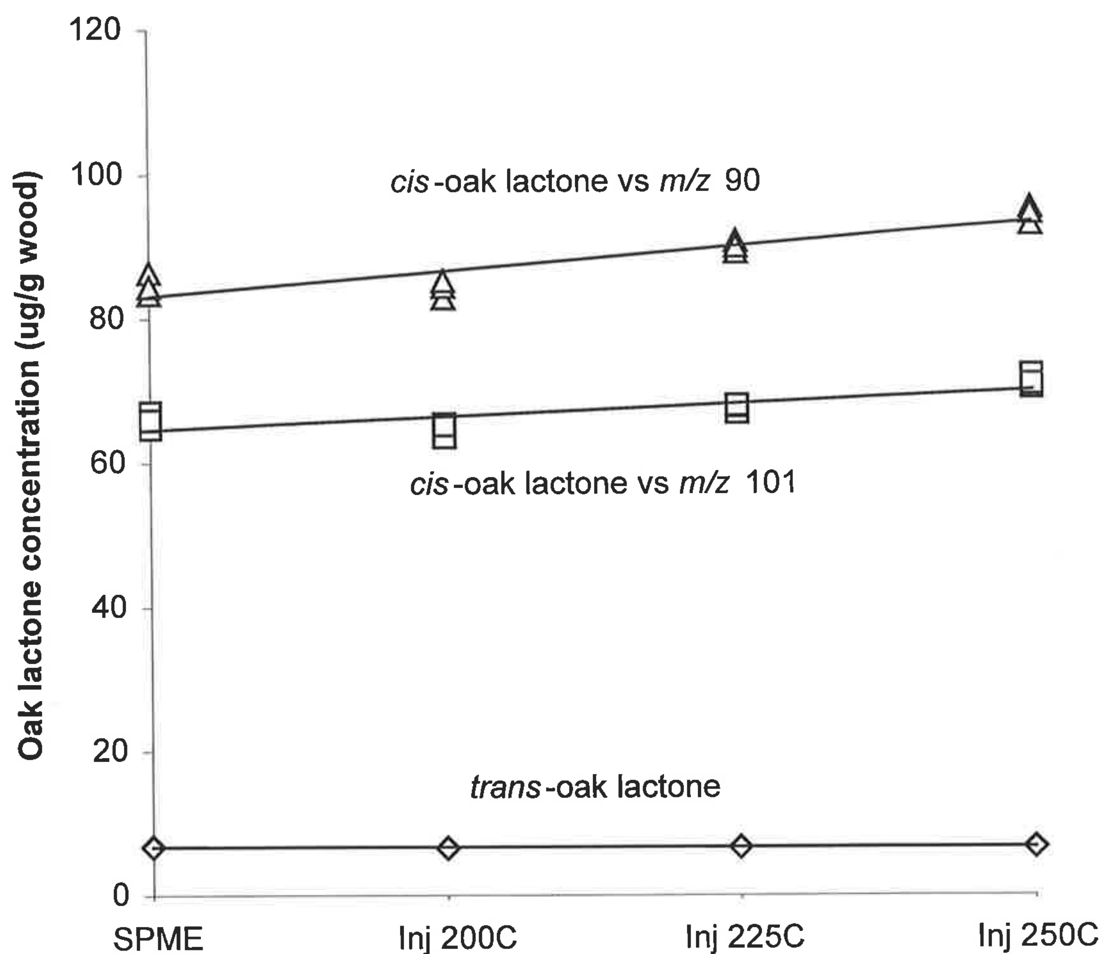


Figure 4.5 Oak lactone concentration measured in unheated *Q. Alba* Fine Grain B – SPME and ether extraction injected at different temperatures

All determinations were done in triplicate.

The increased levels of *cis*-oak lactone shown in figure 4.5 corresponding to injector block temperatures of 225°C or greater shows that significant artefactual generation of *cis*-oak lactone at these temperatures is possible when ether is used as the extracting solvent.

The d_4-*cis*-oak lactone *m/z* 101 co-eluter effect is also evident as it was for figure 4.4.

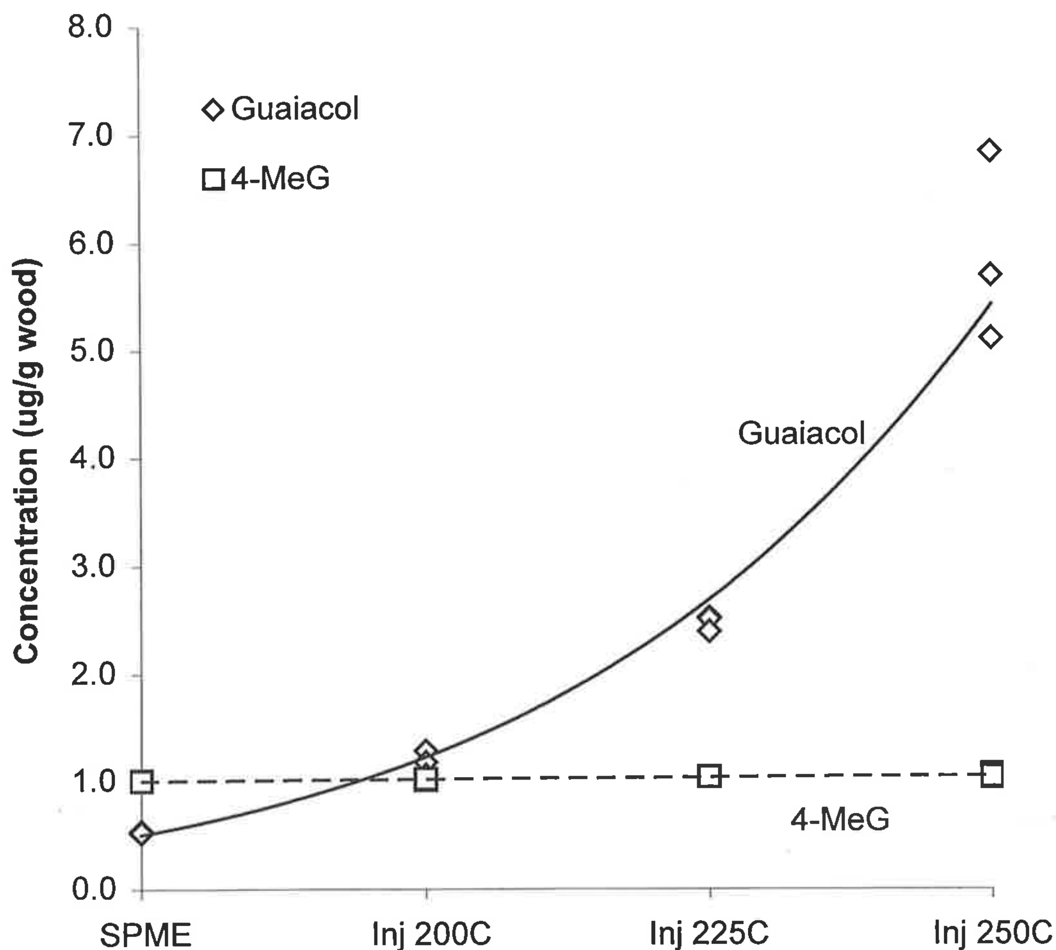


Figure 4.6 Guaiacol and 4-methylguaiacol (4-MeG) concentrations measured in heated Chestnut Oak A – SPME and ether liquid-liquid extraction injected at different injector block temperatures

All determinations were done in triplicate.

Significant (> 99.9%) artefactual generation of guaiacol for ether extractions of this oaked model wine, especially at higher injector block temperatures, can be seen in the above figure. The true result (0.53 μg/g guaiacol and 1.00 μg/g 4-methylguaiacol) obtained by SPME can be exaggerated by more than an order of magnitude (eg. mean of 5.89 μg/g guaiacol, a 1110% factor of increase, with 4-methylguaiacol only increasing 4% to 1.04 μg/g mean, for ether injections at 250°C).

Note that the guaiacol precision is still very good as shown by the closeness of the points on figure 4.6 and the > 99.9% confidences for ether injections at 200°C and 225°C, although the true values are inflated by factors of 221% and 467%, respectively.

Nevertheless, even for this oak extract there was little or no difference in the concentrations measured for both compounds between SPME and liquid-liquid extractions with pentane or 2:1 pentane : ether, as long as the liquid extracts were injected at 200°C (data in Table 4.5, Appendix I, etc). However, this is not the case for Chestnut Oak B as evident from Table 4.5 and Appendix I. Thus, it is important that SPME is used for spot checks to validate analyses involving liquid-liquid extractions of oak shavings, as was done in Chapter 7.

Another example of the importance of extracting solvent is illustrated in figure 4.7.

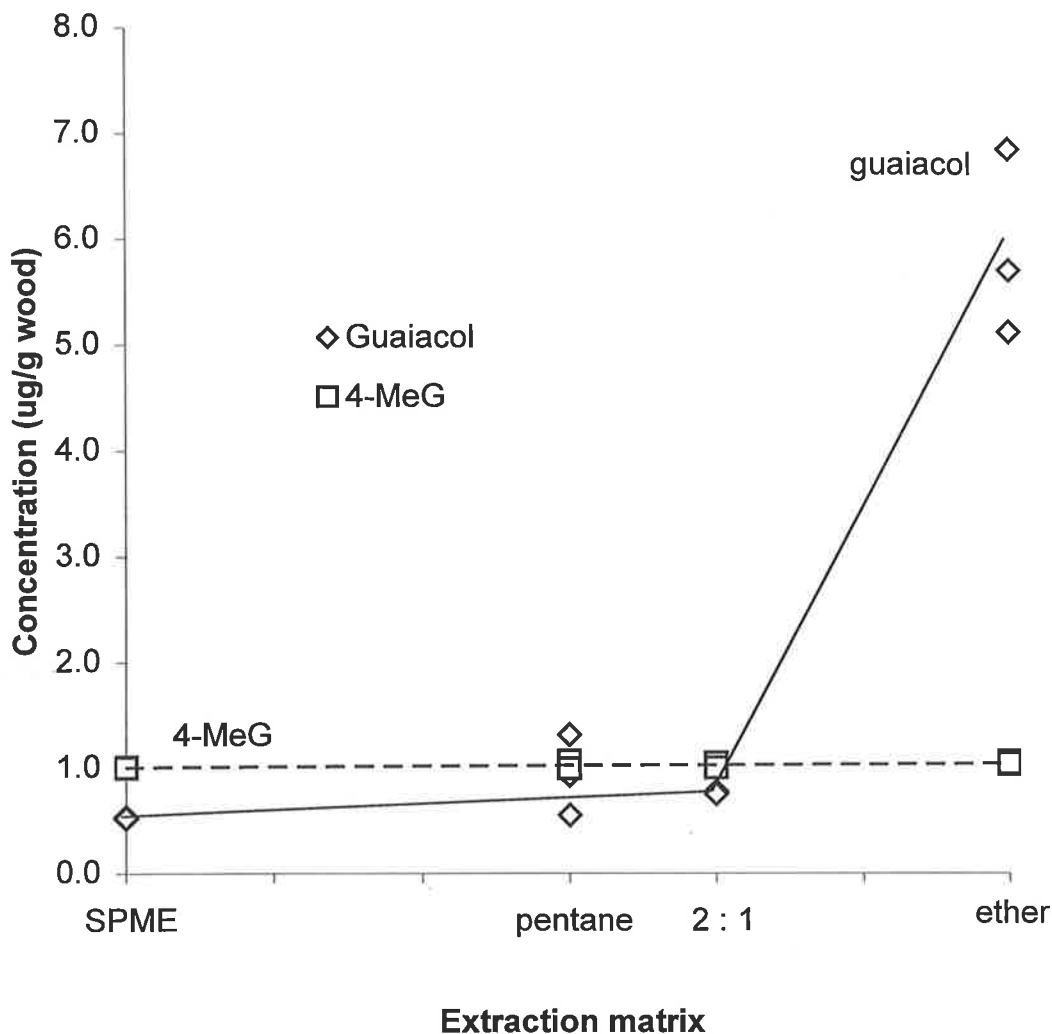


Figure 4.7 Guaiacol and 4-methylguaiacol (4-MeG) concentrations measured in heated Chestnut Oak A – SPME and liquid / liquid extraction with pentane, 2:1 pentane : ether and ether (all liquid injections at 250°C)

All determinations were done in triplicate.

In the above figure, artefactual guaiacol generation is largely solvent dependent. The precision of the analysis was poor for pentane and ether extractions injected at 250°C, hence it is likely that the good precision attained for the 2:1 pentane : ether extracts are coincidence. Again - high injector temperature and ether extraction are clearly significant factors for the artefactual generation of guaiacol.

Even in carefully conducted analyses using suitable isotopically labelled analogues, the possibility of artefactual generation of the analytes of interest during the analysis of oak extracts can never be discarded entirely, unless the analyses are done by SPME or another headspace technique that cannot extract precursors which can then break down to form the analytes in the injector block of the GC. As discussed previously, SPME is unsuited to the analysis of vanillin due to sensitivity problems. In addition, automated SPME equipment is not always available (ie the author of this thesis had no access to such a facility). Thus, in order to analyse a large number of samples (eg this thesis), compromises need to be made. In the majority of cases, liquid-liquid extraction of oak extracts gives acceptable accuracy (+/- 5%), provided that factors conducive to artefact formation are minimised. Hence, the extraction should be rapid (minutes) and not involve any adjustment of the pH of the matrix, a relatively non-polar solvent system should be used (eg 2:1 pentane : ether), and the injector temperature kept to 200°C. These conditions do not generate artefacts during the analyses of red and white wines that have been aged in contact with oak products (as observed by the author 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 (as done in Chapter 7) to ensure that no significant artefactual generation is occurring as a result of the analyses.

The figures (4.6 and 4.7) are a good example of how, even with the benefit of isotopically labelled standards, inaccurate results can be obtained with insufficient attention to analytical detail. This casts doubt upon the accuracy and thus relevance of previous analyses, especially of guaiacol in oak extracts in the literature, as no consideration appears to have been given to these problems. Many extraction methods in the literature use more exhaustive extractions (thus more likely to extract precursors) and polar solvents, eg. dichloromethane (eg. Simpson *et al.* 1986, Boidron *et al.* 1988, Waterhouse and Towey 1994, Towey and Waterhouse 1996a), toluene / ethanol (2:1) (Hale *et al.* 1999). None have the benefit of even one isotopically labelled standard.

4.2.3 Investigations into the effect of pH and air on the quantitative analysis of vanillin and 4-ethylphenol in wine

Guaiacol, 4-methylguaiacol and to a lesser extent, oak lactone, have extractable precursors, hence care needs to be taken in their analysis to avoid artefacts. There are no problems for 4-ethylphenol, which is not derived from oak. Vanillin shows only slight (up to 7%) artefactual generation, even under extreme conditions involving concentrated oak shaving extracts (approx. 100 g shavings per litre of wine) and elevated injector temperatures. Vanillin contains an oxygen atom at the benzylic position, which implies oxidation of lignin could conceivably take place at higher pH values and generate vanillin. To test this possibility the following experiments were conducted.

When the pH of a red wine was adjusted to 6.0, and then internal standard was added, the measured concentration of vanillin in the extract decreased. No significant decrease occurred, however, when the pH adjustment of the wine was done under nitrogen. This implies that the decrease in vanillin when the pH was adjusted to 6.0 was due to oxidative processes, rather than to a 'freezing' of the chemical equilibria between vanillin and possible adducts (eg. acetals).
No change in vanillin concentration was measured if the internal standard was added before the pH of the wine was adjusted to 6.0. Presumably, the rate and extent of oxidative degradation of vanillin and its deuterated analogue are identical.

Without inert gas protection, four red wines were extracted and analysed in duplicate, both with and without adjustment of the pH to 8.5 after the addition of internal standards but prior to extraction and analysis. The results are shown in Table 4.9.

Analysis of extracts of wine without pH adjustment showed good precision (< 5% coefficient of variance, often 0%) between duplicates, but the pH adjusted duplicates were not as precise for vanillin, eg. wine 4 (pH 8.5) has replicates of 259 and 228 μg/L. Each wine gave higher values of vanillin when the pH was adjusted. This increase was greater at higher GC injector block temperatures (up to 250°C), which indicates that at

pH 8.5, precursors to vanillin, possibly oxidized lignins, were being generated, and these then decomposed to vanillin during sample injection.

Table 4.9 Concentrations of vanillin and 4-ethylphenol in oaked red wine – with and without pH adjustment (to 8.5) prior to extraction and analysis

	Vanillin μg/L	4-Ethylphenol μg/L
Wine 1 (pH 8.5)	153	1010
Wine 1 (normal)	113	1010
Wine 2 (pH 8.5)	102	1170
Wine 2 (normal)	82	1160
Wine 3 (pH 8.5)	157	777
Wine 3 (normal)	143	741
Wine 4 (pH 8.5)	244	890
Wine 4 (normal)	197	857

All analyses were done in duplicate – mean results shown.

It is clear from the results in Table 4.9 that following adjustment of the wine pH to 8.5 *after* the addition of the internal standards, vanillin can be generated artefactually. For these four wines an extra 10-50% vanillin was generated. No significant differences were observed for 4-ethylphenol, as expected given its non-lignin origin.

When ether extracts of a red wine plus internal standard were stored for a week at 20°C prior to GC/MS analysis a 10% increase in apparent vanillin concentration was detected. This is probably due to the generation of vanillin from oxidation of the ether soluble precursors within the extract. There may also be competing degradation processes (oxidative or otherwise), but these would effect vanillin and d_3-vanillin at the same rate.

These trials showed that vanillin can be both simultaneously generated and degraded in wine at pH values of 6.0 and above. Thus, extraction methods which involve pH adjustment to basic conditions (eg. Simpson *et al.* 1986, Marsal and Sarre 1987, Chatonnet and Boidron 1988, Boidron *et al.* 1988, Waterhouse and Towey 1994, Towey and Waterhouse 1996a) are likely to give inaccurate results and as such should be avoided. None of these authors demonstrate that this possibility was even considered.

4.2.4 Alternative methods of analysis for guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, oak lactone and vanillin

There is an abundance of analytical methods for the compounds discussed in this thesis. For the most part these are carried out in concert with the analysis of other oak volatiles. The following observations are provided to illustrate comparison of the SIDA method developed here with other methods previously recorded.

Simpson *et al.* (1986) analysed guaiacol in wine and cork using the structurally similar 4-methylguaiacol as internal standard (IS) as a means of investigating guaiacol as a possible cause of cork taint. However, wine aged in oak may already contain 4-methylguaiacol and thus affect the accuracy of results obtained by this method. They obtained good sensitivity with a level of detection below 1 µg/L. Yet, in order to obtain almost complete extraction of guaiacol, their extraction methods are time consuming. Wine (120 mL of a 400 mL sample) was distilled into base, acidified and extracted with dichloromethane (2 x 10 mL), washed with water (2 x 10 mL), dried over sodium sulfate, and then concentrated on two successively smaller Vigreux columns to less than 200 µL organic extract, which was analysed by manual injection onto the GC/MS. Cork was cut into approx. 50 pieces and extracted with Freon 11 (2 x 50 mL), which was then stored overnight at minus 15°C to freeze out any residual water and then concentrated and analysed as above.

Cerny and Grosch (1993) used d_3-guaiacol as an isotopically labelled standard for the accurate quantitation of guaiacol in beef by GC/MS. They did not show a standard addition curve or repeatability data, but they did quantify the relative response factor of d_3-guaiacol versus guaiacol via GC/MS by analysing reference solutions of d_3-guaiacol versus methyl myristate and unlabelled guaiacol versus methyl myristate. Despite the use of d_3-guaiacol as their isotopically labelled IS, their extraction method was very time consuming. No level of detection was given by Cerny and Grosch, but they report quantitation of guaiacol to levels of 2.2 ppb.

Mosedale and Ford (1996) measured *cis*- and *trans*-oak lactone by GC/MS, using δ-decalactone as internal standard. Their method needed only 2 mL of model wine extract and was fairly quick involving three ether / pentane (2:1) liquid-liquid extractions, but still required a concentration step. Their injection into the GC was done at 220°C, but only one ion (*m/z* 99) was monitored for the quantitation of oak lactone, thus no additional confirmation of identity apart from retention time was gained, and thus their quantitation may be inaccurate as co-eluting peaks could be present. Mosedale and Ford mention that they did validate their method with a multi-level calibration graph made from dilutions of a standard solution of oak lactone isomers (Aldrich), however this was not done by spiked standard additions to the matrix analysed, and no correlation data of the calibration graph is given, or even how many points are on the graph. Repeatability data was quoted, but only on the standard solution dilutions and not on the oak wood extracts. It is likely that their analyses of the oak wood extracts was not very repeatable as their method assumes δ-decalactone is equally soluble in ether / pentane as *cis*- and *trans*-oak lactone, and that these compounds evaporate at the same rate during their concentration step.

Hale *et al.* (1999) extracted oak chips with toluene / ethanol (2:1) and measured oak lactone isomers by GC/MS versus methyl laurate as internal standard, which they assume extracted at the same rate from the matrix as both oak lactone isomers. Again, only one ion (*m/z* 99) was monitored for the quantitation of oak lactone, (and only *m/z* 74 for their internal standard) thus no additional confirmation of identity apart from

retention time was gained, and thus quantitation may be made less accurate as co-eluting peaks could be present. The use of ethanol makes their extracting solvent system quite polar and thus conducive to the extraction of precursors, which can form artefacts of the analytes of interest during analysis.

Feuillat *et al.* (1997) measured *cis*- and *trans*-oak lactone in model wine oak extracts. They extracted 200 mL model wine with dichloromethane (3 x 15 mL) and *then* added pentadecane as internal standard. Thus they have no way to measure how much of each oak lactone isomer was extracted from the model wine. Their standard is of use in calibrating their instrumental analysis only (multi-dimensional GC), by the method of Guichard *et al.* 1995 (who did similar analyses on brandy), but even then assumes equal responses for pentadecane as both oak lactone isomers. Feuillat *et al.* (1997) obtained a good detection level of approx. 0.2 μg/L, although they required 200 mL of wine and 45 mL of solvent to obtain this detection limit.

Pérez-Coello *et al.* (1999) soaked oak shavings in model wine for six months then extracted 50 mL of the filtrate with 8 mL dichloromethane, added γ-hexalactone as the only internal standard (IS) and then concentrated under nitrogen to 200 μL. The extract was analysed by GC/MS and 39 volatile components, including furfural, *cis*- and *trans*-oak lactone, vanillin and eugenol were 'quantitated' on the basis of peak areas of the total ion chromatogram (TIC) of a scan run. The one chromatogram they show (presumably their best) has many co-eluting peaks, and no description of how they estimated areas of these co-eluting peaks. They report a level of detection of 0.2 μg/g oak shavings. Their quantitation has many sources of error - Pérez-Coello *et al.* assume that: all the volatiles of interest are extracted into the dichloromethane at the same rate as γ-hexalactone; the relatively polar solvent (dichloromethane) does not extract precursors to the volatiles of interest that may generate those volatiles during the analysis; during their concentration step, γ-hexalactone evaporates at the same rate as all 39 of the volatiles they measured; TIC responses are the same for the same concentrations of 39 different volatiles versus γ-hexalactone; co-elution of peaks does not occur (when the chromatogram they present clearly shows co-eluting peaks).

In a much more solid paper, Boidron *et al.* (1988) measured oak volatiles including furfural, furfuryl alcohol, *cis-* and *trans*-oak lactone, guaiacol, 4-methylguaiacol, 4-ethylguaiacol, eugenol and 4-ethylphenol, versus 3,4-dimethylphenol as internal standard. Despite significant column bleed and poor signal to noise ratio, especially for later eluting compounds, they manage approximate detection limits of 1 μg/L for furfural, furfuryl alcohol and guaiacol, 3 μg/L for 4-methylguaiacol, 40 μg/L for *cis-* and *trans*-oak lactone, 4-ethylphenol and vanillin. Their extraction method for phenols is time consuming and requires 100 mL of wine, 30 g of ammonium sulphate, adjustment of the pH to 8.5, successive dichloromethane extractions, washing with basic solution, reacidification of the aqueous phase, extraction with diethyl ether and injection into the GC at 230°C - factors which are conducive to artefact formation. For oak lactone, Boidron *et al.* use the method of Marsal and Sarre (1987) which uses diethyl ether followed by diethyl ether-hexane (1:1) as extraction solvents, factors which are conducive to artefact formation. The coefficients of variance obtained by Marsal and Sarre (1987) were 8.0% for the *cis* isomer and 9.6% for the *trans* isomer, after reassigning the isomers correctly (Sefton *et al* 1993a).

Towey and Waterhouse (1996a) and Waterhouse and Towey (1994) measure guaiacol, 4-methylguaiacol, *cis-* and *trans*-oak lactone, furfural, furfuryl alcohol, eugenol and 18 other volatile oak compounds versus 3,4-dimethylphenol as internal standard using dichloromethane extracts (in a similar extraction procedure to Boidron *et al.* 1988) and quantitation versus GC/FID (assuming equal FID responses for analytes of the same concentration), with confirmation of identities of GC peaks by GC/MS and injection of known standards. Their level of detection was between 15 and 30 μg/L, depending on the compound analysed. Towey and Waterhouse also take into account that oak volatile extraction is affected by pH and ethanol (Maga 1989a) and show similar pH, ethanol and titratable acidity (TA) contents for all the wines they analysed in both of these papers. They used a carbowax column with an injector block temperature of 200°C; thus, generation of artefacts from precursors should be minimised at this relatively low injector block temperature.

In a previous study of barrel-aged wines, carried out at the Australian Wine Research Institute over several years, (eg Sefton *et al.* 1993a&b, 1990) it had become apparent that, while vanillin in aqueous alcohol extracts of oakwood could be determined with acceptable accuracy by GC/MS, similar analysis of vanillin in red and white wines usually gave anomalously low results, despite exhaustive continuous liquid-liquid extraction of at least 500 mL of wine with three successive aliquots of Freon F11 over 72 h, and a concentration step prior to analysis by GC/MS. Recoveries were variable and often less than 20%. (Sefton and Spillman, unpublished data, see Fig. 4.8). Losses may have occurred due to acetal formation with wine glycols during the extraction of wines with organic solvents and the subsequent concentration of these organic extracts. Such reactions would not take place with aqueous alcohol extracts of oak, which do not contain such glycols.

Problems in measuring vanillin in wines are implied by the omission of data on this potentially important flavour-impact compound from several studies of oak-derived wine volatiles (eg. Chatonnet and Boidron, 1988; Chatonnet *et al.* 1990; Towey and Waterhouse, 1996a&b). In most studies where vanillin in wine extracts has been reported, the concentration has been determined by HPLC (eg. Puech, 1987, 1981, Mosedale and Ford 1996, Mazzoleni *et al.* 1998, Chatonnet and Dubourdieu 1998a, Hale *et al.* 1999).

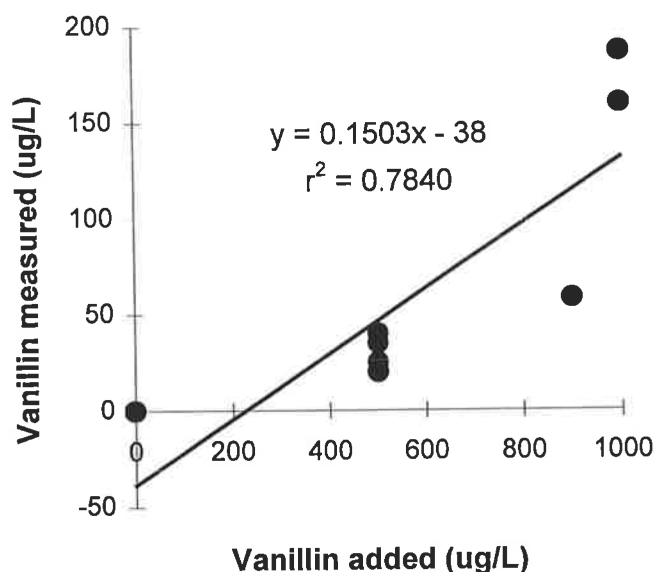


Figure 4.8 Standard addition curve for the analysis of vanillin in wine using continuous liquid-liquid extraction with Freon F11

The above figure (Sefton and Spillman (1993), unpublished data from this laboratory) shows spiked standard additions to a red wine, and the amounts measured by GC/MS by the methodology of Sefton *et al.* (1993a & b) using butylated hydroxy toluene (BHT) as internal standard.

In summary, all the current methods in the literature for the accurate analysis of oak-derived aroma and flavour compounds in wine are more time consuming and less accurate than those described in this thesis. Many literature methods involve steps conducive to the formation of artefacts, such as polar extracting solvents, pH adjustment and high injector block temperatures.

The methodology developed in this thesis is sensitive, rapid, precise and accurate. Combined with automated instrumental analyses, the methodology is used to analyse oak volatiles in large numbers of wine and oak samples in conjunction with industry trials and is offered as a commercial service (http://winetitles.com.au/awri/oak.html). There have also been relevant research applications (eg. Chapters 6 and 7 of this thesis, Spillman *et al.* 1997, Osicka 1997, Spillman *et al.* 1998a, Spillman 1998, Pollnitz *et al.* 1999, Pollnitz *et al.* 2000a-c).

4.3 Vanillyl alcohol and vanillyl ethyl ether

Vanillyl alcohol and vanillyl ethyl ether are not important to wine flavour and aroma. Analytical methods for determining these compounds were developed to aid in studies of vanillin degradation (as discussed in Chapter 1.3.8 and Spillman *et al.* 1998a).

These two compounds are not derived from oak; rather they are formed biologically from vanillin. Thus, the possibility of the artefactual generation of vanillyl alcohol and vanillyl ethyl ether from the thermal degradation of oak extracts in the injector block can be discounted as there is no chemical basis for this assumption.

However, in spiked model wine extracts, injected at temperatures higher than 220°C there was some degradation of vanillyl alcohol into vanillin, 4-methylguaiacol and vanillyl ethyl ether. The extent of degradation and the relative ratio of compounds formed were variable and depended on concentration of the extracts, ethanol content and injector block temperature and cleanliness. More 4-methylguaiacol was formed at higher temperatures (data not shown).

It was important to prepare the vanillyl alcohol and d_3-vanillyl alcohol spiking solutions in water, so that no ether formation to the corresponding vanillyl ethyl ether occurred. Likewise, the vanillyl ethyl ether and d_3-vanillyl ethyl ether spiking solutions were prepared in ethanol for the opposite reason.

Vanillyl alcohol was determined by a separate extraction and analysis technique using phenyl solid phase extraction (SPE) cartridges as described in section 4.1.2. If the multi-oak compound methodology (section 4.1.1) was used there was poor recovery of vanillyl alcohol (> 100 μg/L level of detection) due to its high water solubility. Attempts to load more wine than 1 mL of wine onto the cartridge, or washing with water after sample loading and prior to dichloromethane elution, resulted in significantly lower recoveries of vanillyl alcohol. Attempts involving C18 SPE cartridges or liquid-liquid extraction with organic solvents (pentane, pentane / ether, ether, ethyl acetate or dichloromethane) yielded less than detectable amounts of vanillyl alcohol and d_3-vanillyl alcohol).

Vanillyl ethyl ether was measured by similar methodology to the multi-oak assay (section 4.1.1), using ether as the extracting solvent, as detailed in section 4.1.2.

There is no analysis for vanillyl ethyl ether reported in the literature (excluding work by the author). In all studies (excluding work involving the author) where vanillyl alcohol has been reported, the concentration has been determined by HPLC (eg. Chatonnet 1991).

With the use of d_3-vanillyl alcohol and d_3-vanillyl ethyl ether as internal standards, vanillyl alcohol and vanillyl ethyl ether can now be rapidly and accurately quantified in wine, using the methods developed in section 4.1.2. Combined with automated instrumental techniques these methods have enabled assays for these two compounds on a scale that was hitherto unattainable. Applications of these new methods can be found in Spillman *et al.* (1998a).

Chapter 5

Application of the analytical method for chloroanisoles to wine and cork samples

This chapter deals with applications of the analysis for chloroanisoles developed in Chapter 3. Wine, whole corks and ground corks are analysed.

5.1 MATERIALS AND METHODS

5.1.1 Sampling of wines from a wine assessment course

Thirty participants in the Advanced Wine Assessment Short Course, conducted by The Australian Wine Research Institute in April 1995, were asked to assess all wines presented during the course for cork taint. The participants were experienced wine assessors, employed in production, marketing and journalism in the Australian wine industry. Two bottles of each wine were presented on any one occasion, with each bottle being assessed by a group of 15 participants. All participants were asked to take note of any wines thought to be affected by cork taint. Following discussion of the wines, those bottles of wine considered by three or more assessors to be affected by recognisable cork taint, as well as the corks from those bottles, were collected and stored at -10°C prior to analysis. All wines so collected were from 750 mL bottles. Some wines were presented on up to three occasions (ie. requiring up to six bottles). All wines assessed at the course were purchased from commercial outlets. The wines analysed to give the data in Tables 5.2 and 5.3 were obtained directly from the producers and had not been previously subjected to any sensory screening.

5.1.2 Cork dissection into four portions – old and young, inside and outside

In some straight wine corks, it is possible to tell which hemicircular half of the upright cylinder was closest to the outer circumference of the tree. This half, representing the older bark, was determined by firstly inspecting the growth rings within the cork and then looking at the two faces on each side of the diameter parallel to the growth rings. The older bark contains a greater density of lenticels, which are breathing holes that permit gas-exchange between the metabolically active cells below the bark and the

atmosphere (Esau 1967). Five corks were cut in half, lengthwise along the plane of the growth rings, resulting in one half-cylinder consisting of the younger bark and one half-cylinder of the older bark. Then the half corks were divided into an inside fraction and an outside fraction. This was done by slicing two approx. 2 mm thick hemicircular discs off the ends of the half cork, and then applying cuts to remove the outermost curved layer, approx. 2 mm thick, all along the length of the half cork. The cut-off cork fractions from each individual half cork were grouped as one fraction and weighed, as was the single inside half cylinder. Thus, four fractions were made from each cork, referred to as "Old Inside", "Old Outside", "Young Inside" and "Young Outside" from hereon. The corks were dissected with a scalpel using sterile carbon steel surgical blades and a thoroughly cleaned glass surface for each dissection. The blades were changed frequently and thoroughly clean forceps were used to handle the cork pieces for each portion of the cork. Every reasonable effort was made to avoid cross contamination.

These twenty cork fractions were individually weighed, ground and analysed by the method (described in Chapter 3).

5.1.3 Qualitative analysis of all possible mono- to penta- chloroanisoles

The analysis for all 19 possible chloroanisoles was done in the same manner as that for the five common chloroanisoles (Chapter 3), except that in SIM runs the ions monitored were: *m/z* 112, 127, 142, 144 for 2-chloroanisole, 3-chloroanisole and 4-chloroanisole; *m/z* 161, 176, 178 for 2,3-dichloroanisole, 2,4-dichloroanisole, 2,5-dichloroanisole, 2,6-dichloroanisole, 3,4-dichloroanisole and 3,5-dichloroanisole; *m/z* 195, 210, 212 for 2,3,4-trichloroanisole, 2,3,5-trichloroanisole, 2,3,6-trichloroanisole, 2,4,5-trichloroanisole, 2,4,6-trichloroanisole, 3,4,5-trichloroanisole; *m/z* 215, 217 for d_5-2,4,6-trichloroanisole (Internal Standard); *m/z* 203, 229, 246 for 2,3,4,6-tetrachloroanisole and 2,3,5,6-tetrachloroanisole; *m/z* 203, 231, 244, 246 for 2,3,4,5-tetrachloroanisole and *m/z* 237, 265, 280 for pentachloroanisole. Unlabelled chloroanisole reference standards were purchased from Aldrich Chemical Company.

5.2 RESULTS AND DISCUSSION

5.2.1 Analysis of commercial wines and the distribution of chloroanisoles between the wines and the corks taken from the bottles

Of 374 bottles of wine presented to, and discussed by, the participants of the Advanced Wine Assessment Short Course conducted by The Australian Wine Research Institute in April 1995, 4.8% (ie. 18 bottles out of the 374) were considered by at least 20% of the participants to be affected by recognisable cork taint (Table 5.1). Among the dry and sparkling white wines, 6.8% (15 bottles out of 220) were seen as tainted, as were 2% of the reds (3 bottles out of 154). A similar proportion (5.6%) had been assessed as affected by cork taint when 720 bottles of wine were presented over two courses conducted the previous year (P. Leske, personal communication, 1995).

The 18 wine samples assessed as tainted were analysed (as described in chapter 3) for the five chloroanisoles commonly associated with contaminated corks (2,4-DCA, 2,6-DCA, TCA, TeCA and PCA; Simpson 1990). The results are also shown in Table 5.1. All of the wine samples contained TCA at a concentration of 1 ng / 750 mL or greater. Two out of two bottles of one of the wines presented were also considered as tainted by the participants, but as there was no apparent bottle-to-bottle variation in the level of taint, this could not be attributed with confidence to the cork, and so these samples have not been included in Table 5.1. No chloroanisoles were detected in either of these two wines.

Table 5.1 Chloroanisoles detected in wines assessed as affected by cork-taint and in the corks taken from the bottles

Sample	Taint score [a]	Concentration (ng/750 mL of wine or ng/cork) [b] TCA		Other Chloroanisoles	
		Wine	Cork	Wine	Cork
White wines					
1 Riesling	3	1	1	n.d.	2,4-DCA (1), 2,6-DCA (1)
2 Chardonnay	4	1	3	n.d.	n.d.
3 Chardonnay	5	1	8	n.d.	n.d.
4 Semillon	6	2	12	n.d.	2,6-DCA (5)
5 Botrytised Semillon	6	4	10	n.d.	n.d.
6 Chardonnay [c]	7	1	5	n.d.	2,6-DCA (tr)
7 Chardonnay [c]	7	1	4	n.d.	2,4-DCA (1)
8 Sparkling Riesling	7	3	15	n.d.	PCA (4)
9 Riesling [d]	10	2	20	PCA (2)	PCA (28)
10 Riesling [d]	10	4	12	PCA (2)	TeCA (tr), PCA (7)
11 Chardonnay	12	2	18	2,4-DCA (2)	2,4-DCA (2), 2,6-DCA (2)
12 Riesling	14	3	21	n.d.	2,6-DCA (12)
13 Riesling [d]	15	2	8	n.d.	TeCA (tr), PCA (4)
14 Riesling [d]	15	4	14	PCA (3)	PCA (4)
15 Riesling	15	28	41	2,4-DCA (16)	2,4-DCA (5)
Red Wines					
16 Cabernet	7	4	33	n.d.	n.d.
17 Shiraz	10	3	7	2,4-DCA (2)	n.d.
18 Cabernet	12	5	1	n.d.	2,6-DCA (2)

n.d., none detected; tr., trace (< 1 ng)

[a] Number of assessors (out of 15) who detected taint

[b] Concentration is given to the nearest ng.

[c] Samples 6 and 7 were two bottles of the same wine. Four other bottles of this wine were presented but were not seen as tainted.

[d] Samples 9, 10, 13 and 14 were four bottles of the same wine. Two other bottles of this wine were presented but were not seen as tainted.

The sensory threshold for TCA in a Pinot Noir wine is 1.4 ng/L according to Duerr (1985) and 4.0 ng/L in a dry white wine according to Amon *et al.* (1989). This is in good agreement with data obtained by Liacopoulos *et al.* (1999) who determined sensory thresholds from 2.0 to 4.6 ng/L in a range of red and white dry wines.

Published sensory thresholds are essentially approximate mean values of the thresholds for individuals within a group, and there can be considerable variation between individuals in their sensitivity to taints and other aromas (Land 1989). Some panellists will be able to detect a substance at a concentration well below that of the group threshold, while others will only be able to detect the aroma at a much higher concentration. Thus, although the concentration of TCA in some of the wines listed in Table 5.1 was below the range of published (group) thresholds, it is probable that this compound would nevertheless have been detected in all of the wines by at least a minority of panellists.

The TCA contamination in the wines was attributed to the cork, since variation in apparent taint between bottles of the same wine was observed in every case. Analysis of the corks from these bottles (Table 5.1) and subsequent experiments by Capone *et al.* (1999) confirmed this assumption.

With the exception of bottles 6 and 7, those wines containing less than 2 ng/L of TCA were seen as tainted by the smallest number of participants. All other wines were seen as tainted by at least 40% of the assessors. There appears to be no precise correlation between the concentration of TCA and the number of assessors judging the wine as tainted, but this is not surprising, given that a considerable variation of wine style is represented and that two separate panels of judges carried out the assessments. In addition, other compounds derived from the cork (discussed in Chapter 1.3) may also have contributed to the perceived taint in some bottles such as 6 and 7.

For two of the wines, six bottles of each were presented and more than one bottle was seen as tainted (Table 5.1). These were a Chardonnay (bottles 6 and 7) and a Riesling (bottles 9, 10, 13 and 14). Further bottles of these wines were analysed for the five chloroanisoles to determine whether this was likely to be a result of chance, or whether these wines were sealed with a batch of corks with a high frequency of contamination. Of the 12 additional bottles of this Chardonnay analysed, none contained any detectable chloroanisoles. Four out of fourteen bottles of the Riesling, however, were contaminated with TCA. The data are shown in Table 5.2. All but one of the 14 corks from the Riesling contained TCA, but in a majority of cases (9) this had not leached into the wine.

Similar results were obtained when, during the course of this study, 12 bottles, and the corks from those bottles, of a Pinot Noir wine considered by its producers to be affected by cork taint, were submitted to this laboratory for analysis. The results are given in Table 5.3. All of the corks and one third (4 out of 12) of the wines contained TCA.

Thus, while cork taint may be no more than a nuisance for wine producers and consumers on most occasions, contamination of corks by TCA can sometimes cause severe problems for individual producers.

Whole corks were extracted in this early work. Later experiments showed that grinding cork was required for a more complete extraction (section 5.2.2, Capone *et al.* 1999, Howland *et al.* 1997). Hence, the cork values shown in these tables are an underestimate of the true value within the cork.

Table 5.2 **Chloroanisoles detected in 14 bottles of a Riesling wine and in the corks taken from the bottles** [a]

	Concentration (ng/750 mL of wine, or ng/cork)			
	TCA		Other Chloroanisoles	
Sample	**Wine**	**Cork**	**Wine**	**Cork**
1	n.d.	16	n.d.	PCA(9)
2	n.d.	15	n.d.	2,4-DCA(8), PCA(18)
3	n.d.	3	n.d.	n.d.
4	3	6	PCA(14)	PCA(22)
5	5	17	n.d.	PCA(13)
6	n.d.	6	n.d.	PCA(13)
7	n.d.	n.d.	n.d.	PCA(14)
8	n.d.	7	n.d.	PCA(20)
9	n.d.	7	PCA(11)	PCA(18)
10	n.d.	8	n.d.	PCA(3)
11	n.d.	3	n.d.	PCA(2)
12	2	9	PCA(5)	PCA(6)
13	2	2	PCA(2)	2,4-DCA(tr), 2,6-DCA(tr), PCA(1)
14	n.d.	3	PCA(6)	2,4-DCA(tr), 2,6-DCA(1), PCA(3)

n.d., none detected; tr., trace (< 1 ng).

[a] Bottles were selected at random and had been sealed for twelve months.

Table 5.3 **Chloroanisoles detected in 12 bottles of a Pinot Noir wine and in the corks taken from the bottles [a]**

	Concentration (ng/750 mL of wine, or ng/cork)			
	TCA		Other Chloroanisoles	
Sample	**Wine**	**Cork**	**Wine**	**Cork**
1	10	15	PCA(2)	2,4-DCA(tr), TeCA(1), PCA(8)
2	12	29	n.d.	2,4-DCA(22), TeCA(tr), PCA(5)
3	n.d.	12	n.d.	TeCA(1), PCA(9)
4	n.d.	8	PCA(8)	TeCA(tr), PCA(4)
5	n.d.	14	n.d.	PCA(10)
6	n.d.	12	PCA(3)	2,4-DCA(tr), TeCA(1), PCA(6)
7	10	10	PCA(2)	2,4-DCA(tr), TeCA(tr), PCA(5)
8	n.d.	16	PCA(5)	2,4-DCA(tr), TeCA(1), PCA(4)
9	n.d.	5	PCA(3)	PCA(1)
10	2	12	n.d.	PCA(3)
11	n.d.	12	PCA(5)	PCA(3)
12	n.d.	10	n.d.	2,4-DCA(5), PCA(2)

n.d., none detected; tr., trace (< 1 ng).

[a] Bottles were selected at random and had been sealed for two years.

Origins of TCA

In order to shed light on the origin of the TCA in the wines and corks, a detailed search for those chloroanisoles accompanying TCA was carried out by analysing extracts of corks from the tainted wines listed in Tables 5.1 - 5.3 for all 19 possible chloroanisoles containing one to five chlorine atoms. The results are also listed in Tables 5.1 - 5.3. From past experience (troubleshooting in this laboratory), chloroanisoles can be found in corks from tainted wines in higher amounts than in the tainted wines themselves, and this was also the case in this study.

The observation that TCA in contaminated corks sampled from the wine assessment course co-occurred with 2,4- and/or 2,6-DCA in the majority of samples, suggests that chlorine bleaching, and/or the use of TCP-based biocides in the forest or during transportation of corks, may be responsible for much of the cork taint observed in the Australian wine industry (2,4-DCP and 2,6-DCP are products of incomplete chlorination of phenol under mild conditions). However, TeCA and PCA were also observed in some contaminated corks - particularly those that were part of a batch with an apparent high proportion of contamination. The origin of the taint in these corks can therefore be ascribed to the use of polychlorinated biocides.

The ratio of the chloroanisoles in the corks varied considerably, but this is perhaps not surprising given that there are several biochemical mechanisms whereby micro-organisms can remove chlorophenols from their environment (Neidleman and Geigert 1986), and that these mechanisms (eg. methylation) need not operate with the same efficiency on different chlorophenols (Whitfield et al. 1991a & b). The ratio of the chloroanisoles in the corks could also be altered by the various treatments to which corks may be subjected during processing.

In none of the 44 cork samples was a chloroanisole observed with a substitution pattern other than that derived by chemical chlorination of phenol, ie only the five common chloroanisoles (2,4-DCA, 2,6-DCA, 2,4,6-TCA, 2,3,4,6-TeCA and PCA) were detected. This indicates that microbial reductive dechlorination of polychlorophenols was not an important source of chloroanisoles in any of the tainted wines, as this process usually leads to products with selective retention of meta-chlorine atoms (see Introduction, section 1.3.3).

Distribution of chloroanisoles between wines and corks

As also observed by Amon *et al.* (1989), there was considerable variation in the distribution of TCA between wines and corks (Tables 5.1 - 5.3). In a majority of cases, little if any of the TCA in the corks appears to have been absorbed by the wine. This is particularly evident in those samples listed in Tables 5.2 and 5.3, which, unlike the wines from the Advanced Wine Assessment Course (Table 5.1), were selected at random, and not by sensory screening. Similar data has been obtained for additional corks examined using the method described herein, but carried out subsequently to the work described in this chapter (Capone *et al.* 1999, Howland *et al.* 1997), using fully ground corks rather than whole corks.

There was also considerable variation among bottles in the distribution of other chloroanisoles between wine and cork and indeed, in the distribution of different chloroanisoles in any one sample of bottled wine. This is best illustrated by the data in Table 5.3. The corks from all twelve bottles contained both TCA and PCA. In two of these bottles (2 and 10) only TCA was absorbed by the wine, while in five others (4, 6, 8, 9 and 11) only PCA was transferred. Three wine samples (3, 5 and 12) contained neither TCA nor PCA, while the remaining two (1 and 7) contained both.

These results indicate that the location of chloroanisoles in corks prior to use varies from one cork to another, and that, in any one cork, the location of different chloroanisoles also varies. The results support the view of Amon and Simpson (1986) that, only when contaminated parts of the cork are in direct contact with, or close to, the wine (or headspace above the wine), does transfer of chloroanisoles take place. If this is the case, then whether or not a contaminated cork ultimately contaminates a wine may depend, *inter alia*, on which end happens to be inserted into the bottle.

5.2.2 The distribution of TCA within the cork

Five corks were selected from a batch found to give rise to an unacceptably high incidence of cork taint when used as bottle closures. These five corks were cut in half, and then the outer thickness of approx. 2 mm was cut out of each cork to make four fractions from each cork, referred to as "Old Inside", "Old Outside", "Young Inside" and "Young Outside".

These twenty cork fractions were extracted and analysed by the method, giving the results shown in Table 5.4. In contrast to the previous section, cork was ground to give a more complete extraction. The outside 2 mm of the cork was denser than the inside portion (presumably as a result of the chlorination treatment); hence, concentrations of chloroanisoles in the table are expressed as ng per gram of cork in each fraction.

Table 5.4 Location of chloroanisoles in five corks

All chloroanisole measurements in ng per gram of cork

Cork number and segment.	mass (g)	2,6-DCA (ng/g)	2,4-DCA (ng/g)	2,4,6-TCA (ng/g)	2,4,6-TCA as % total
1, Young Inside	1.1866	< 0.4	4.2	5.9	11
1, Young Outside	0.4509	ND	4.1	22.6	44
1, Old Inside	1.3324	ND	< 0.4	4.2	8
1, Old Outside	0.6579	< 0.8	3.5	19.2	37
2, Young Inside	1.1666	ND	< 0.2	0.9	11
2, Young Outside	0.6950	ND	< 0.7	2.3	28
2, Old Inside	0.8164	ND	< 0.6	1.5	19
2, Old Outside	0.4435	ND	< 1.1	3.4	42
3, Young Inside	1.2514	ND	ND	2.8	8
3, Young Outside	0.6142	ND	< 0.8	5.7	17
3, Old Inside	0.9044	ND	< 0.6	3.3	10
3, Old Outside	0.5189	< 1.0	< 1.0	22.2	65
4, Young Inside	0.7447	ND	< 0.7	0.9	15
4, Young Outside	0.5490	ND	ND	3.1	50
4, Old Inside	1.2627	ND	< 0.4	0.8	13
4, Old Outside	0.7333	ND	ND	1.4	23
5, Young Inside	0.7693	ND	ND	ND	0
5, Young Outside	0.5234	ND	< 1.0	1.9	32
5, Old Inside	0.7608	ND	< 0.6	0.7	12
5, Old Outside	0.6637	ND	< 0.8	3.4	57

No other chloroanisoles were detected in the corks.

ND means not detected above the detection limit of 0.1 ng / cork.

"2,4,6-TCA as % total" refers to the percentage (rounded to the nearest %) of TCA in that fraction of the cork as compared to the total amount for that cork.

There was some interference from coeluting peaks in the DCA regions, thus "< 0.5" means that DCA may or may not be present underneath a co-eluting peak of area equivalent to a 0.5 ng concentration of that particular DCA.

2,4,6-TCA was measured in every cork (in Table 5.4), accompanied by smaller amounts of 2,4- and 2,6-DCA. Thus, it is likely that at some stage during production the corks had been contaminated with 2,4,6-trichlorophenol (TCP) accompanied by smaller amounts of 2,4- and 2,6-dichlorophenol (DCP). The most likely stage is during the chlorination of cork to bleach it. Indeed phenol (a lignin degradation product) chlorinates in this manner forming TCP and smaller amounts of 2,4- and 2,6-DCP, in a manner similar to the synthesis of d_2-TCP (Scheme 2.1). Microorganisms then methylated these phenols to the corresponding anisoles as a means of detoxifying their environment (Amon and Simpson 1986, Amon *et al.* 1989, Sponholz and Muno 1994).

There was more TCA on the outside of the cork than on the inside for both the older and younger halves ($p< 0.001$ for both). For the five corks analysed there was no significant difference between the older and younger bark. In another experiment, Howland *et al.* (1997) sliced corks from this batch transversely to the growth rings into six-equal sized discs and subsequent analysis showed that there was no significant difference between the amount of TCA found at either end to the middle of the corks. Thus the TCA measured in the outer portions of the cork is a function of the whole outer surface rather than just the end discs, hence the higher TCA levels measured on the outside of the corks in Table 5.4 indicates that at least the final step in the formation of TCA in the corks followed the punching stage of cork production rather than the processing of whole cork slabs. It is not clear at which stage following punching this formation of TCA occurred, ie. it could have been during storage and/or transportation, however as the TCA appears to be localised in the cork in a random fashion, rather than showing similar amounts on all sides, contamination of these corks by aerial TCA migration (Barker *et al.* 2000) from external sources seems highly improbable.

Whatever the source of TCA (hence it's chlorophenolic precursors) in these corks, better control of the microbiological growth on the corks following the punching stage would probably have prevented this instance of cork taint.

Chapter 6

Application of the analytical method for guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans*- and *cis*-oak lactone and vanillin – Red wine composition

In Chapter 4, new methodology for the accurate determination of important oak volatiles in wine by stable isotope dilution analysis was developed. In this chapter, the method is applied to several trials relevant to the wine industry. Although oak derived flavour is important in some white wines (eg Chardonnay), this chapter focuses on red wines which are a more challenging matrix to analyse and have higher concentrations of *cis*-oak lactone and 4-ethylphenol, the two most important aroma and flavour compounds associated with oak barrel maturation.

Vanillyl alcohol and vanillyl ethyl ether are not included in this or the following chapter as applications of these analyses have been carried out in conjunction with others in this laboratory and reported (Spillman *et al.* 1998a, Osicka 1997), and as such are not discussed further in this thesis.

6.1 MATERIALS AND METHODS

Determination of guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans*-oak lactone, *cis*-oak lactone and vanillin in red wine aged in French and American oak barrels of different ages and in the same red wine aged in new French and American oak barrels from different cooperages.

Coopers were instructed to give all these barrels a medium toast at the time of firing. No sulfur dioxide was added prior to fermentation. All the red wines were fermented on skins for 5-7 days and went through partial malolactic fermentation prior to going into the barrel. All wines had matured in the barrels for approximately eight months. Wine samples (5 mL) were taken and the internal standards added on site, prior to transport back to the lab and subsequent extraction and analysis by the method (Chapter 4). The

1998 Cabernet Sauvignon / Shiraz had pH=3.52, titratable acidity = 7.2 g/L (expressed as tartaric acid), alcohol = 14.3%, total SO_2 approx 35 ppm. The 1998 Shiraz aged in American barrels had pH=3.55, titratable acidity = 6.8 g/L (expressed as tartaric acid), alcohol = 14.3%, total SO_2 approx 40 ppm. The 1998 Shiraz aged in French barrels had pH=3.53, titratable acidity = 6.7 g/L (expressed as tartaric acid), alcohol = 13.7%, total SO_2 approx 40 ppm. The 1998 Shiraz used in the cooperage trial (section 6.2.2) had pH=3.53, titratable acidity = 6.7 g/L (expressed as tartaric acid), alcohol = 13.7%, total SO_2 approx 40 ppm.

6.2 RESULTS AND DISCUSSION

6.2.1 Determination of guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans*-oak lactone, *cis*-oak lactone and vanillin in red wine aged in French and American oak barrels of different ages.

The concentrations of guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans*-oak lactone, *cis*-oak lactone and vanillin in red wines aged for eight months in new and used French and American oak barrels are shown in Table 7.1.

Effects of barrel re-use

There are possible sources of variation in the new oak composition, with the probability that the wood comes from different lots and is not necessarily strictly coopered in the same manner. Notwithstanding these possible variations, the concentrations (Table 6.1) of guaiacol, 4-methylguaiacol, *trans*- and *cis*-oak lactone and vanillin were always higher in new barrels than they were in barrels previously used (excluding shaved and refired barrels). The only exception was vanillin in the French oak used twice, which was not significantly different to the vanillin in the new oak, due to the large co-efficient of variance (21%) for vanillin in those samples. This may be due to differences in the depth of heating of the barrels when they were toasted by the cooper (Spillman *et al.* 1996, Spillman 1998).

Table 6.1 Oak volatile concentrations[a] in red wines aged in French and American oak barrels of different ages

1998 Cabernet Sauvignon / Shiraz

Oak	Previous Barrel Use	Guaiacol µg/L (cv%)	4-MeG µg/L (cv%)	4-EP µg/L (cv%)	4-EG µg/L (cv%)	*trans*-OL µg/L (cv%)	*cis*-OL µg/L (cv%)	Vanillin µg/L (cv%)
American	new	26 (8%)	9 (13%)	201 (6%)	24 (4%)	64 (6%)	572 (6%)	348 (13%)
American	used once	17 (6%)	6 (7%)	391 (8%)	35 (10%)	44 (3%)	420 (2%)	268 (4%)

1998 Shiraz

Oak	Previous Barrel Use	Guaiacol µg/L (cv%)	4-MeG µg/L (cv%)	4-EP µg/L (cv%)	4-EG µg/L (cv%)	*trans*-OL µg/L (cv%)	*cis*-OL µg/L (cv%)	Vanillin µg/L (cv%)
American	used twice	22 (13%)	5 (16%)	563 (5%)	31 (3%)	37 (7%)	316 (11%)	357 (7%)
American	3 times	19 (2%)	3 (14%)	505 (9%)	31 (10%)	29 (15%)	259 (7%)	273 (12%)
American	4 times	21 (7%)	4 (17%)	555 (11%)	31 (13%)	29 (3%)	278 (4%)	266 (12%)
American (re-shaved & fired)	4 times	18 (30%)	4 (26%)	95 (29%)	5 (30%)	28 (28%)	267 (29%)	342 (30%)

1998 Shiraz

Oak	Previous Barrel Use	Guaiacol µg/L (cv%)	4-MeG µg/L (cv%)	4-EP µg/L (cv%)	4-EG µg/L (cv%)	*trans*-OL µg/L (cv%)	*cis*-OL µg/L (cv%)	Vanillin µg/L (cv%)
French	new	22 (11%)	8 (13%)	540 (24%)	34 (19%)	128 (44%)	217 (15%)	341 (12%)
French	used once	15 (6%)	3 (14%)	500 (8%)	28 (6%)	64 (30%)	162 (16%)	243 (5%)
French	used twice	18 (16%)	4 (28%)	499 (15%)	33 (14%)	102 (7%)	164 (12%)	390 (21%)
French	3 times	17 (5%)	3 (19%)	514 (8%)	24 (6%)	33 (11%)	65 (5%)	172 (15%)
French (re-shaved & fired)	3 times	19 (4%)	5 (9%)	401 (14%)	23 (10%)	77 (7%)	125 (5%)	360 (5%)

[a] Concentrations shown are the mean from the analysis of five barrels and are expressed in µg/L of red wine

4-MeG = 4-methylguaiacol, 4-EP = 4-ethylphenol, 4-EG = 4-ethylguaiacol, OL = oak lactone, cv = coefficient of variance

The possible variations in the composition of the wood used for barrel making and coopering technique are not a concern for 4-ethylphenol as it is not extracted from oak (Chapter 7). Wine composition as well as microorganisms in the barrel can affect the concentration of 4-ethylphenol (Chapter 1.3.4.3 and references cited therein). It is not certain that all the 4-ethylphenol in the wine was generated only in the barrel, as measurements of the wine were not taken prior to storage. Nevertheless, some differences between groups of barrels were observed which is consistent with the observation of Chatonnet *et al.* (1992b and 1995) that 4-ethylphenol was not present immediately following fermentation but was formed slowly in the wine during barrel maturation. As shown in Table 6.1, the Cabernet Sauvignon / Shiraz red wine blend matured in new American oak had significantly less 4-ethylphenol and 4-ethylguaiacol (201 μg/L and 24 μg/L respectively) than the same blend aged in barrels previously used once (391 μg/L and 35 μg/L respectively). On the other hand, for the group of barrels (French and American) in which the Shiraz wine was stored the previous usage of the barrels had no significant effect on the amount of 4-ethylphenol or 4-ethylguaiacol found.

Effects of shaving and re-firing

Shaving and re-firing the American oak barrels previously used four times resulted in a substantial decrease (of over 80%) in the concentration of 4-ethylphenol and 4-ethylguaiacol in the stored wine. This difference is > 99.9% significant.
No significant differences were observed for guaiacol, 4-methylguaiacol, vanillin and the oak lactone isomers.

Shaving and re-firing the French oak barrels previously used three times resulted in a small but significant reduction of the mean 4-ethylphenol concentration by approx. 20% (from 514 μg/L to 401 μg/L). The small loss of 4-ethylguaiacol, which was present in all the barrels at low concentration, was not statistically significant.

The loss of 4-ethylphenol resulting from shaving and refiring can be attributed to a reduction of the microbiological load on the inner surface of the barrel, prior to use (Chatonnet *et al.* 1992b and 1995).

Shaving and re-firing the French oak barrels previously used three times generated increased levels of guaiacol (17 μg/L to 19 μg/L, > 99% significant), 4-methylguaiacol (3 to 5 μg/L, > 99.9% significant), *trans*-oak lactone (33 μg/L to 77 μg/L, > 99.9% significant), *cis*-oak lactone (65 μg/L to 125 μg/L, > 99.9% significant) and vanillin (172 μg/L to 360 μg/L, > 99.9% significant). Apart from guaiacol, these increases are all approximately double. Guaiacol has been previously identified in hydrolysates of glycosidic fractions from Cabernet Sauvignon and Merlot grape samples (Sefton, 1998), so it is possible that some of the guaiacol in the wines was also derived from the grape.

6.2.2 The concentrations of guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans*-oak lactone, *cis*-oak lactone and vanillin measured in the same red wine aged in new French and American oak barrels from different cooperages.

As a separate trial, the same 1998 Shiraz red wine was aged in 19 different types of new 300 litre oak barrels of either French or American origin, made by 12 different cooperages. Wine from two barrels of each type was analysed by the method, except for one French oak barrel that had no duplicate. Results are shown in Appendix III and further summarised in Table 6.2.

Only *cis*- and *trans*-oak lactone showed significant (> 99.9%) differences in the concentrations measured in wine aged in French oak compared to American oak. There was more *cis*-oak lactone in American than French oak. The average ratios of *cis* : *trans* were 9.4 : 1 for American oak and 1.5 : 1 for French. These results confirm the usefulness of the *cis* : *trans* ratio in indicating oak origin and are in good agreement with the previous section and the literature (Waterhouse and Towey 1994, Guichard *et al.* 1995, Towey and Waterhouse 1996a&b, Swan 1997, Maga 1996).

The highest *cis* : *trans* ratio (in American oak) reported by others in the literature was approx. 8 : 1 by Guichard *et al.* 1995. Waterhouse and Towey 1994, Towey and Waterhouse 1996a&b, Swan 1997, Maga 1996 are more in the range of 4-8 : 1 *cis* / *trans* for American oak. The reason the ratio is higher in this study is most likely due to analytical reasons. On most GC columns, the more volatile *trans*-oak lactone gives approximately a 20% larger peak area than *cis*-oak lactone for the same concentration of analyte. Quantitative analyses that do not take this relative response factor into account by calibrating with mixtures containing known concentrations of each pure isomer (eg. all the work reported in the literature excluding work done by this author) will discriminate against *cis*-oak lactone and thus show lower *cis* / *trans* ratios than are actually present. Furthermore these literature methods do not utilise SIDA or demonstrate any knowledge of co-eluting peaks.

Fine versus medium grained oak

Of the 19 different types of barrels represented in Table 6.2 and Appendix III, eight types were suitable for the comparison of fine versus medium grained oak, as all the other known variables (eg cooperage, medium toast level, oak origin, wine) were as similar as possible. Only one cooperage had American oak of fine and medium grain. The only significant (> 95%) difference observed was for *trans*-oak lactone, which had a mean concentration of 58 µg/L in the four medium grained barrels compared to a mean concentration of 82 µg/L in the two fine grained barrels. This same cooperage also showed significant differences between their fine and medium grain French oak. Guaiacol and 4-methylguaiacol showed > 95% significant differences between their mean concentrations in the four fine grain barrels (33 µg/L guaiacol, 19 µg/L 4-methylguaiacol) and their mean concentrations in the two medium grain barrels (19 µg/L guaiacol, 8 µg/L 4-methylguaiacol). Vanillin showed a > 99.9% significant difference with 486 µg/L (fine grain mean) versus 288 µg/L (medium grain mean). A second cooperage also showed a > 99% significant difference for vanillin in French oak with 483 µg/L (mean of two fine grain barrels) versus 421 µg/L (mean of two medium grain barrels). A third cooperage showed a > 99.9% significant difference in guaiacol concentration between two fine grain (mean 29 µg/L) and two medium grain French barrels (24 µg/L).

Table 6.2 The concentrations of oak volatiles in the same red wine aged in French and American oak from different cooperages

	Guaiacol µg/L (cv%)	4-MeG µg/L (cv%)	4-EP µg/L (cv%)	4-EG µg/L (cv%)	*trans*-OL µg/L (cv%)	*cis*-OL µg/L (cv%)	Vanillin µg/L (cv%)
American Oak Mean (n = 16)	33 (32%)	11 (39%)	542 (21%)	35 (19%)	63 (22%)	595 (23%)	532 (31%)
French Oak Mean (n = 21)	26 (24%)	13 (39%)	492 (18%)	30 (13%)	140 (54%)	208 (36%)	424 (21%)

All concentrations are in µg/L of red wine.

4-MeG = 4-methylguaiacol, 4-EP = 4-ethylphenol, 4-EG = 4-ethylguaiacol, OL = oak lactone, cv = co-efficient of variance

When the combined data from all the fine grain French oak barrels is compared to that of the medium grain French oak barrels, the mean concentrations of guaiacol (30 μg/L fine, 22 μg/L medium) and vanillin (490 μg/L fine, 396 μg/L medium) were higher in the fine grain barrels with > 95% significance.

Only 4-methylguaiacol showed a significant (> 95%) difference between all the fine grain oak barrels, both French and American combined, and all the medium grain oak barrels. The mean 4-methylguaiacol of all the fine grain barrels was 16 μg/L compared to 12 μg/L for the medium grain barrels.

In all cases where a significant difference was observed, the concentration was higher in the fine grain oak. It could be assumed that the fine grain wood is denser and there is a higher concentration of extractable flavour volatiles per gram of the oak wood. However, many coopers and winemakers presume that fine grain oak is of better quality than medium grain and this may have affected how the barrels were toasted or treated, eg. the higher vanillin concentration in the fine grain oak could be due to increased depth of heating during toasting as observed by Spillman *et al.* 1996, Spillman 1998.

There were no significant differences observed for *cis*-oak lactone, 4-ethylphenol or 4-ethylguaiacol, the three compounds most important to the aroma and flavour of these wines (section 6.2.3). Feuillat *et al.* (1997) also observed no significant difference in the concentrations of *cis*- and *trans*-oak lactone between fine and medium grained French oak, however they did observe significant correlations for the concentrations of total ellagitannins.

6.2.3 Oak volatile analysis of 61 different commercial bottled red wines

61 different commercially available bottled red wines of various vintages were analysed for guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans*- and *cis*-oak lactone and vanillin by the method (developed in Chapter 4). These wines were from different regions throughout Australia and were of four main varieties – Cabernet Sauvignon, Merlot, Pinot Noir and Shiraz. Results are shown in Tables 6.3 and 6.4.

Some of the differences observed in oak volatile concentration according to variety can be readily explained, for example Shiraz and Cabernet Sauvignon had more *cis*-oak lactone than Pinot Noir because these wines generally have more exposure to American oak and new oak during the process of winemaking. Too few (nine) Merlot wines were analysed to make relevant comparisons, considering the range of concentrations observed in these Merlots.

Guaiacol and 4-methylguaiacol

Some of the differences observed between varieties can not be readily explained in terms of oak composition alone, such as the significantly higher guaiacol in Shiraz wines. This suggests a grape-derived precursor of guaiacol is present in Shiraz grapes at a significantly higher concentration than in the other grape varieties analysed. Indeed, Sefton (1998) found guaiacol present in hydrolysates of glycosidic fractions from Cabernet Sauvignon and Merlot grapes that had never had any oak contact, although Sefton (1998) did not analyse Shiraz. Guaiacol was found in every red wine. The concentrations found varied between 10 μg/L in a Pinot Noir up to 72 μg/L in a 1996 Shiraz, with a mean of 28 μg/L.

Table 6.3 Oak assay on 61 different commercial bottled red wines[a]

Wine Cabernet Sauvignon	Level of Oak Compounds (μg/L) Guaiacol	4-MeG	*trans*-OL	*cis*-OL	*Ratio cis/trans*	Vanillin	4-EP	4-EG	*Ratio 4-EP/4-EG*
1986 Cabernet Sauvignon	19	6	93	178	*1.9*	154	2060	240	*8.6*
1992 Cabernet Sauvignon	23	10	192	320	*1.7*	188	267	45	*5.9*
1992 Cabernet Sauvignon	17	2	54	192	*3.6*	69	2450	141	*17.4*
1992 Cabernet Sauvignon	16	3	75	183	*2.4*	70	851	61	*14.1*
1993 Cabernet Sauvignon	18	6	92	157	*1.7*	89	2150	226	*9.5*
1994 Cabernet Sauvignon	28	8	31	410	***13.3***	113	594	47	*12.6*
1994 Cabernet Sauvignon	26	5	32	434	***13.6***	91	697	69	*10.1*
1994 Cabernet Sauvignon	39	9	93	417	*4.5*	253	1840	187	*9.8*
1994 Cabernet Sauvignon	21	2	102	206	*2.0*	71	1530	104	*14.7*
1994 Cabernet Sauvignon	14	6	82	232	*2.8*	200	683	72	*9.5*
1994 Cabernet Sauvignon	33	9	70	779	***11.1***	127	688	96	*7.1*
1994 Cabernet Sauvignon	20	5	21	213	***10.3***	86	518	45	*11.4*
1994 Cabernet Sauvignon	26	5	74	428	*5.8*	336	1130	145	*7.8*
1994 Cabernet Sauvignon	16	2	19	149	***8.0***	77	1870	115	*16.3*
1995 Cabernet Sauvignon	19	9	23	214	***9.3***	137	1130	295	*3.8*
1995 Cabernet Sauvignon	40	26	74	143	*1.9*	230	1240	134	*9.3*
1995 Cabernet Sauvignon	20	5	48	887	***18.5***	143	834	73	*11.4*
1997 Cabernet Sauvignon	21	4	127	183	*1.4*	57	1910	129	*14.8*
Cabernet Sauvignon Average	**23**	**7**	**72**	**318**	***6.3***	**138**	**1250**	**124**	***10.1***
Standard Deviation =	8	5	43	214	*5.2*	77	659	73	*3.6*
95% CI =	20 - 26	4 - 10	52 - 92	219 - 417	*3.9 - 8.7*	103 - 173	950-1550	90 - 158	*8.4 - 11.8*
99% CI =	18 - 28	4 - 10	46 - 98	188 - 448	*3.1 - 9.5*	92 - 184	850-1650	80 - 168	*7.9 - 12.3*
99.9% CI =	17 - 29	3 - 11	38 - 104	152 - 484	*2.3 - 10.3*	79 - 197	740-1760	68 - 180	*7.3 - 12.9*
Overall Average (over 4 varieties)	**28**	**7**	**64**	**280**	***4.9****	**156**	**795**	**99**	***8.0***
					**Median shown*				

a) No two bottles are the same product

Bold cis/trans ratio indicates use of mainly American oak

Wine Merlot	Level of Oak Compounds (µg/L) Guaiacol	4-MeG	*trans*-OL	*cis*-OL	*Ratio cis/trans*	Vanillin	4-EP	4-EG	*Ratio 4-EP/4-EG*
1988 Merlot	15	4	44	118	*2.7*	39	604	62	*9.7*
1991 Merlot	17	6	103	160	*1.6*	130	2200	437	*5.0*
1994 Merlot	26	6	89	267	*3.0*	179	1820	165	*11.0*
1995 Merlot	17	8	67	136	*2.0*	133	528	61	*8.7*
1995 Merlot	16	3	36	263	***7.3***	83	2100	232	*9.1*
1995 Merlot	42	15	37	395	***10.7***	314	1280	113	*11.3*
1995 Merlot	12	2	trace	trace	*not applicable*	126	23	6	*3.8*
1996 Merlot	18	11	26	74	*2.8*	172	2	2	*1.0*
1996 Merlot	16	5	51	197	*3.9*	155	324	72	*4.5*
Merlot Average	**20**	**7**	**57**	**201**	***4.2***	**148**	**987**	**128**	***7.1***
Standard Deviation =	9	4	27	103	*3.1*	76	879	137	*3.6*
95% CI =	14 - 29	4 -10	39 - 75	133 - 269	*2.0 - 5.4*	98 - 198	412-1560	38 - 218	*5.7 - 9.5*
99% CI =	12 - 28	3 - 11	35 - 81	112 - 290	*1.3 - 7.1*	83 - 213	232-1742	10 - 246	*4.0 - 10.2*
99.9% CI =	10 - 30	2 - 12	27 - 87	88 - 314	*0.5 - 8.9*	65 - 231	22-1952	nd - 279	*3.1 - 11.1*

Wine Pinot Noir	Level of Oak Compounds (µg/L) Guaiacol	4-MeG	*trans*-OL	*cis*-OL	*Ratio cis/trans*	Vanillin	4-EP	4-EG	*Ratio 4-EP/4-EG*
1986 Pinot Noir	15	4	53	80	*1.5*	44	3	1	*3.0*
1991 Pinot Noir	31	8	119	266	*2.2*	214	51	21	*2.4*
1992 Pinot Noir	19	4	112	191	*1.7*	148	114	28	*4.1*
1994 Pinot Noir	21	4	47	103	*2.2*	127	1240	421	*2.9*
1995 Pinot Noir	10	1	2	34	***17.0***	34	58	13	*4.5*
1995 Pinot Noir	19	8	127	186	*1.5*	172	202	98	*2.1*
1995 Pinot Noir	15	3	139	221	*1.6*	173	1560	311	*5.0*
1995 Pinot Noir	28	12	99	159	*1.6*	111	32	23	*1.4*
1995 Pinot Noir	25	4	48	80	*1.7*	107	83	35	*2.4*
1995 Pinot Noir	34	10	164	306	*1.9*	347	193	121	*1.6*
1996 Pinot Noir	11	2	16	71	*4.4*	85	197	39	*5.1*
1996 Pinot Noir	42	17	6	83	***13.8***	96	498	126	*4.0*
1996 Pinot Noir	32	3	66	104	*1.6*	134	169	28	*6.0*
Pinot Noir Average	**23**	**6**	**77**	**145**	***4.1***	**138**	**338**	**97**	***3.4***
Standard Deviation =	10	5	53	84	*5.1*	81	492	128	*1.5*
95% CI =	18 - 28	3 - 9	48 - 106	100 - 190	*1.3 - 6.9*	94 - 182	71 - 605	27 - 167	*2.8 - 4.2*
99% CI =	16 - 30	3 - 9	39 - 115	85 - 205	*1.2 - 7.0*	80 - 196	nd - 689	6 - 188	*2.4 - 4.4*
99.9% CI =	14 - 32	4 - 10	28 - 126	69 - 221	*0.4 - 8.8*	64 - 212	nd - 787	nd - 214	*2.1 - 4.7*

Wine	Level of Oak Compounds (µg/L)								
Shiraz	Guaiacol	4-MeG	*trans*-OL	*cis*-OL	*Ratio cis/trans*	Vanillin	4-EP	4-EG	*Ratio 4-EP/4-EG*
1987 Shiraz	33	6	90	828	***9.2***	290	633	75	*8.4*
1987 Hermitage	53	7	70	794	***11.3***	119	275	75	*3.7*
1988 Hermitage	45	6	68	750	***11.0***	156	186	51	*3.6*
1989 Shiraz	49	3	53	90	*1.7*	84	82	12	*6.8*
1993 Shiraz	40	4	48	621	***12.9***	129	115	12	*9.6*
1994 Hermitage	27	3	58	103	*1.8*	171	1310	84	*15.6*
1994 Shiraz	40	14	99	533	*5.4*	296	282	66	*4.3*
1994 Shiraz	48	12	77	630	***8.2***	252	1580	99	*16.0*
1995 Shiraz	28	7	40	153	*3.8*	154	232	38	*6.1*
1995 Shiraz	67	16	98	622	*6.3*	232	407	67	*6.1*
1995 Shiraz	17	1	4	79	***19.8***	57	113	6	*18.8*
1995 Shiraz	21	2	5	78	***15.6***	121	709	31	*22.9*
1995 Shiraz	19	2	trace	102	***> 102 to 1***	96	844	57	*14.8*
1995 Shiraz	29	7	38	295	***7.8***	132	258	29	*8.9*
1995 Shiraz	27	7	29	219	***7.6***	312	524	78	*6.7*
1995 Shiraz	39	5	trace	15	***> 15 to 1***	155	283	28	*10.1*
1996 Shiraz	17	3	trace	39	***> 39 to 1***	50	169	14	*12.1*
1996 Shiraz	26	4	26	298	***11.5***	142	572	44	*13.0*
1996 Shiraz	44	9	40	462	***11.6***	328	1390	161	*8.6*
1996 Shiraz	49	10	32	409	***12.8***	285	72	9	*8.0*
1996 Shiraz	72	18	59	553	***9.4***	364	2660	350	*7.6*
Shiraz Average	**38**	**7**	**52**	**365**	***11.0****	**187**	**605**	**66**	***10.1***
Standard Deviation =	15	5	28	274	*> 21.3*	95	647	75	*5.1*
95% CI =	31 - 45	5 - 9	40 - 64	248 - 482		146 - 228	328 - 882	34 - 98	*8.9 - 12.3*
99% CI =	29 - 47	4 - 10	36 - 68	211 - 519		134 - 187	241 - 969	24 - 108	*8.2 - 13.0*
99.9% CI =	27 - 49	4 - 10	32 - 72	168 - 562		119 - 255	140 - 1070	12 - 120	*7.4 - 13.8*

Bold cis/trans ratio indicates use of mainly American oak

**Median shown*

Overall Average (over 4 varieties)	28	7	64	280	4.9*	156	795	99	8.0

Table 6.4 Statistical summary of oak volatile assay of 61 different commercial bottled red wines

Mean concentration in μg/L

	Cabernet	Merlot	Pinot	Shiraz	All
Guaiacol	23^{b**}	20^{b**}	23^{b*}	38^{a}	28
4-MeG	7^{a}	7^{a}	6^{a}	7^{a}	7
4-EP	1250^{a}	987^{ab}	338^{b**}	605^{b*}	795
4-EG	124^{a}	128^{a}	97^{a}	66^{a}	99
***trans*-OL**	72^{a}	57^{a}	77^{a}	52^{a}	64
***cis*-OL**	318^{a*}	201^{ab}	145^{b}	365^{a**}	280
Vanillin	138^{a}	148^{a}	138^{a}	187^{a}	156

a,b represent different groups. Values in the same row with the same superscript letter were not significantly different at the 5% level.

* difference is > 95% significant

** difference is > 99% significant

Guaiacol	Shiraz > Cabernet Sauvignon (99%), Merlot (99%), Pinot Noir (95%)
4-MeG	No significant differences
4-EP	Cabernet Sauvignon > Pinot Noir (99%), Shiraz (95%)
4-EG	No significant differences
***trans*-OL**	No significant differences
***cis*-OL**	Shiraz (99%), Cabernet Sauvignon (95%) > Pinot Noir
Vanillin	No significant differences

Low concentrations of guaiacol were typically accompanied by lower concentrations of 4-methylguaiacol. 4-Methylguaiacol was also in every wine analysed, its concentration ranging from 1 μg/L in a Pinot Noir and a Shiraz, up to 26 μg/L in a Cabernet Sauvignon, with a mean of 7 μg/L. There was always more guaiacol than 4-methylguaiacol present in the wines, the relative amounts ranged from about 1.5 : 1 (typical of extraction from heated oak shavings in model wine – see Chapter 7) up to 11 : 1 (indicating that some guaiacol is also derived from the grape {Sefton 1998}), with a median ratio of approx. 4 : 1.

Oak lactone

The oak lactone isomers were also found in all of the wines, although one Merlot had only a trace (< 1 μg/L) of each isomer. As oak lactones have only been detected in wines that have been matured in oak barrels or treated with other oak products, it would appear that virtually all 61 wines had some oak contact during their production. Both the absolute amounts of both oak lactone isomers and the *cis / trans* ratio varied widely in the wines. The highest level of *cis*-oak lactone found was 887 μg/L in a Cabernet Sauvignon. The overall mean concentrations observed were 64 μg/L *trans*-oak lactone and 280 μg/L *cis*-oak lactone. Three 1995 Shiraz wines had only trace (< 1 μg/L) *trans*-oak lactone, but contained 102 μg/L, 15 μg/L and 39 μg/L *cis*-oak lactone respectively. Such high amounts of *cis*-oak lactone without corresponding levels of *trans*-oak lactone have never been reported in the literature. The median ratio of *cis / trans* observed in the Shiraz wines was 11 : 1, indicating the dominance of new American oak in Australian Shiraz winemaking. This median value is used for Shiraz in the table because a mean would be vastly weighted towards the three highest values. In several wines, the ratio of *cis*- to *trans*-oak lactones is 10 or higher. This may simply indicate that the range of ratios in oak products is somewhat higher than indicated by previous trials. Alternatively, it is possible that, during bottle maturation, *cis*-oak lactone is generated faster than the *trans*-isomer from precursor forms that have also been extracted from the oak. However, such a mechanism for enhancing oak lactone concentration in wine has not so far been demonstrated.

Wines that had a *cis / trans* ratio of greater than 7 to 1 (shown in bold in Table 6.3) were arbitrarily deemed to have been made with American oak only, with the higher ratios coupled to the higher absolute amounts attributed to new American oak. Wines with a *cis / trans* ratio less than 2.5 to 1 were probably made only in contact with French oak, as was the case for 10 of 13 Pinot Noir wines. Two of the remaining three Pinot Noir wines were most likely matured in previously used American oak, as the total amounts of oak lactone observed are low. Wines with a *cis / trans* ratio between 2.5 and 7.0 were most likely aged in contact with a mixture of both oak types (eg the remaining Pinot Noir wine).

Using these ratio definitions, the following observations can be made for each variety...

Cabernet – 7/18 wines had American oak only, 7 had French only, 4 were blends
Pinot Noir – 2/13 wines had used American oak, 10/13 French, one blend
Merlot – 2/9 wines had American oak only, one wine had < 1 μg/L of each isomer, the rest were blends
Shiraz – 16/21 wines had American oak only (most had new American oak), 2 had French only, and 3 blends

Vanillin

Vanillin was present in all 61 reds, varying in concentration from 34 μg/L in a 1995 Pinot Noir up to 364 μg/L in one of the youngest (1996) Shiraz wines, with an average concentration of 156 μg/L across all four varieties. There were no significant differences in vanillin concentration between the varieties.

4-Ethylphenol and 4-ethylguaiacol

4-Ethylphenol was detected in every red wine. The concentrations found in the wines varied between 2 μg/L in a Merlot and 2660 μg/L in a Shiraz, with a mean

concentration of 795 µg/L. (The highest level of 4-ethylphenol observed in this laboratory whilst analysing "problem wines" is approx. 8000 µg/L.) There was usually more 4-ethylphenol present than any other of the oak volatiles assayed, but there were several exceptions. 4-Ethylguaiacol was also found in every red wine analysed, varying in concentration from 1 µg/L (in a Pinot Noir) up to 437 µg/L (in a Merlot) with a mean concentration of 99 µg/L. Within the wines of each variety, a wide range of concentrations was observed, consistent with the results of Chatonnet *et al.* (1992b & 1995) who have demonstrated the importance of winemaking practices on the formation of 4-ethylphenol and 4-ethylguaiacol.

Although not enough wines were analysed to make a comprehensive investigation into the relationship between variety and 4-ethylphenol concentration, some trends were nevertheless observed. The mean concentration of 4-ethylphenol found in the Cabernet Sauvignon wines (1250 µg/L) was greater than that of Shiraz (605 µg/L) with > 95% confidence and Pinot Noir (338 µg/L) with > 99% confidence. Too few Merlots were analysed to make any significant comparisons, especially considering that the level of 4-ethylphenol found in the nine Merlots analysed ranged from 2 µg/L up to 2200 µg/L. There was no significant difference in 4-ethylguaiacol concentration between varieties.

The ratio of 4-ethylphenol to 4-ethylguaiacol also varied from wine to wine, as shown in Table 6.3. The average ratio was 10.1 : 1 for Cabernet Sauvignon and Shiraz, 7.1 : 1 for Merlot and 3.4 : 1 for Pinot Noir. The mean ratio of Pinot Noir was lower than that of Merlot with > 95% significance, and lower than that of Cabernet Sauvignon and Shiraz with > 99.9% significance.

Apart from a Merlot which had 2 µg/L of each compound, the lowest ratio of 4-ethylphenol to 4-ethylguaiacol observed was 32 : 23 = 1.4 : 1 in a 1995 Pinot Noir. The highest ratio was 709 : 31 = 23 : 1 in a 1995 Shiraz.

Goldberg *et al* (1998) measured the concentration of *p*-coumaric acid, the precursor to 4-ethylphenol, in single-variety red wines from various countries. The range in *p*-coumaric acid concentration can be compared to that observed for 4-ethylphenol in the

bottled red wines studied here. Among the Australian wines, *p*-coumaric acid was lowest in Pinot Noir as compared to Shiraz and Cabernet Sauvignon. In Californian wines, Pinot Noir was also equally lowest in *p*-coumaric acid (along with Zinfandel) and in South African wines, Pinot Noir had the lowest *p*-coumaric acid of the six varieties assayed. Indeed, Goldberg *et al* (1998) found Pinot Noir generally had low levels of *p*-coumaric acid across all varieties and countries.

Thus, although wine maturation conditions are paramount in determining the concentration of 4-ethylphenol and 4-ethylguaiacol to be found in commercial wines (Chatonnet *et al* 1992b and 1995), genetic or cultural factors may also be influential. Further experiments are necessary to test this hypothesis.

6.2.4 Sensory significance of the results

Cis-oak lactone is of primary importance in the oak-derived flavour of these wines. Synthetic racemic *cis*-oak lactone has a perception threshold of 67 µg/L in white wine (Chatonnet 1991). In all the wine in the barrels measured in section 6.2.2, except those aged in French oak barrels previously used three times, more than double the threshold was measured in all of the wines, with the highest concentrations coming from new American oak barrels. A high proportion of Australian red wine makers appear to use new American oak for their high quality red wines, especially for Shiraz, as shown from the commercial wine data (section 6.2.3).

Chatonnet *et al.* (1992b), using the methodology of Boidron *et al.* (1988), determined individual detection thresholds of 605 µg/L and 110 µg/L for 4-ethylphenol and 4-ethylguaiacol respectively in a red wine. However, when combined in a typical ratio of 10:1 the detection threshold was 334 µg/L 4-ethylphenol plus 34 µg/L 4-ethylguaiacol in red wine (Chatonnet *et al.* 1995). Thus the aroma of the wines in all the barrels in Tables 6.1, 6.2 and Appendix III is likely to be moderately affected by the concentrations of 4-ethylphenol and 4-ethylguaiacol. In the 61 commercial wines, however, 4-ethylphenol and 4-ethylguaiacol were more important as there were

generally higher concentrations of both. Although not enough wines were analysed to draw firm conclusions about the relationship between variety and 4-ethylphenol/4-ethylguaiacol concentration, some trends were nevertheless observed. A high concentration of 4-ethylphenol was particularly evident with many of the Cabernet Sauvignon wines. A larger proportion of Shiraz and Pinot Noir wines contained sub-threshold amounts of 4-ethylphenol, as indicated by the median values, although high concentrations (> 1000 μg/L) were still detected in some of these wines.

Vanillin can be perceived as such above 320 μg/L in red wine (Boidron *et al.* 1988). Thus the aroma of the wines in all the barrels in Tables 6.1, 6.2 and Appendix III should only be slightly affected by the concentration of vanillin. Lower concentrations were observed in most of the 61 commercial bottled wines analysed in Table 6.3. Thus, vanillin should have little impact on the aroma and flavour of most of these wines.

Synthetic racemic *trans*-oak lactone has a perception threshold of 460 μg/L in white wine (Chatonnet 1991). Guaiacol has a perception threshold of 20 μg/L in white wine (Simpson *et al.* 1986), but can only be perceived as 'smoky' above 75 μg/L in red wine (Boidron *et al.* 1988). 4-Methylguaiacol has a similar detection threshold of 65 μg/L in red wine (Boidron *et al.* 1988). The aroma of the wines in Tables 6.1, 6.2, Appendix III and Table 6.3 is therefore unlikely to be strongly affected by the measured concentrations of guaiacol, 4-methylguaiacol and *trans*-oak lactone. However, it is possible that these compounds have a subtle and complexing effect on the wine aroma.

Chapter 7

Application of the analytical method for guaiacol, 4-methylguaiacol, *trans*- and *cis*-oak lactone and vanillin – Oak composition

Although 4-ethylphenol and 4-ethylguaiacol were important to red wine flavour (as shown in Chapter 6), they were found to be of little interest in the analysis of oak. No 4-ethylphenol was detected in any of the oak extracts analysed in this chapter. Only low levels (< 10 μg/L) of 4-ethylguaiacol were found in extracts of toasted oak, and none was found in unheated oak. As such, results of the analyses of these two compounds are not shown nor discussed further in this chapter.

7.1 MATERIALS AND METHODS

Due to time constraints, the number of samples analysed, and the absence of automated SPME equipment, it was not possible to analyse all of the oak extracts in this chapter by SPME. In addition, as discussed in Chapter 4.2.1.4, SPME gave poor sensitivity for vanillin. Nevertheless, numerous spot checks were done in order to verify that there was no artefactual generation of the volatiles of interest (eg guaiacol) as a result of the analyses. In all cases, there was no significant difference between the concentrations of the analytes determined by SPME as compared to the liquid-liquid extracts. No significant generation of any of the analytes was observed in any sample.

7.1.1 Effects of air and temperature on the formation of guaiacol, 4-methylguaiacol, *trans*- and *cis*-oak lactone and vanillin in heated oak shavings

Fine shavings (approx. 20 mm x 1 mm thick needles) were prepared from two oak wood samples, a stave of American oak (*Quercus alba*, from a local supplier in South

Australia) and a stave of French oak (*Q. sessilis* from Troncais) using a Skil 94H 400W rotary plane. The species of the oak samples was determined by the suppliers, but has not been independently confirmed by the author. Twenty-eight subsamples of the oak shavings (*circa* 10 g each, exact masses for each replicate were French oak control #1 – 7.516g, #2 – 6.80g, French oak argon 25°C #1 – 12.76g, #2 – 8.60g, French oak air 25°C #1 – 4.71g, #2 – 5.926g, French oak argon 175°C #1 – 16.42g, #2 – 12.63g, French oak air 175°C #1 – 4.04g, #2 – 5.20g, French oak argon 225°C #1 – 5.63g, #2 – 8.48g, French oak air 225°C #1 – 13.99g, #2 – 10.00g, American oak control 25°C #1 – 8.70g, #2 – 8.53g, American oak argon 25°C #1 – 10.76g, #2 – 10.54g, American oak air 25°C #1 – 11.53g, #2 – 9.95g, American oak argon 175°C #1 – 13.08g, #2 – 7.59g, American oak air 175°C #1 – 7.62g, #2 – 7.02g, American oak argon 225°C #1 – 7.31g, #2 – 12.04g, American oak air 225°C #1 – 17.90g, #2 – 10.24g) were placed in all glass reagent bottles with firmly sealing glass stoppers. Four of the samples, two from each oak type were controls and had no ultrasound or heat treatment. Six of the samples for each oak type were purged with argon (Air Liquide, ultra high purity) then ultrasonicated for 5 min. then purged again with argon then ultrasonicated for 5 min. and then purged a third time with argon, followed by a third ultrasonication for 5 min. and then a final purge with argon. Two of these samples were stored at 25°C; two were heated at 175°C and two at 225°C. The remaining six samples for each oak type were treated in exactly the same manner, except under air instead of argon. All heating was done with the same gas cover (ie air or argon) in a Carbolite constant temperature (+/- 1°C) oven fitted with a Eurotherm digital controller (Medos, Adelaide, Australia) for 2 h after which the samples were allowed to cool. Each treatment for each oak type was done in duplicate. All 28 samples of shavings were individually soaked in 50 mL of model wine (10% ethanol, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid) at 25°C for 1 week, after which time the shavings were removed by filtration through glass wool. Separate aliquots (5 mL) from these 28 solutions were extracted in duplicate by liquid-liquid extraction with pentane-diethyl ether (2:1, 2 mL) and analysed by the method. Extraction rate studies indicated that 1 week was sufficient time to extract the compounds from the oak shavings (see below).

7.1.2 Evolution of volatile compounds by extraction from oak shavings and formation in solution

Fine shavings (1 mm thickness) were prepared using a Skil 94H 400W rotary plane from 21 oak wood samples, from different regions in France and America. (Details are given in Appendix IV). The species of some American oak samples was determined by the suppliers, but has not been independently confirmed by the author. Subsamples of the oak shavings (*circa* 5-10 g each, exact masses given in Appendix IV) were heated in air at 200°C in a constant temperature oven (+/- 1°C) for 2 h after which the samples were allowed to cool. All 21 samples of shavings were soaked in 50 mL of model wine (10% ethanol in water, adjusted to pH 3.0 with potassium hydrogen tartrate and conc. hydrochloric acid) at 25°C for 1 week, after which time separate 5 mL aliquots from these 21 solutions were extracted by liquid-liquid extraction with pentane-diethyl ether (2:1, 2 mL) and analysed by the method. The shavings were removed by decantation and filtration through glass wool and the filtrates were heated (in sealed vessels) at 80°C for 10 days after which time separate 5 mL aliquots from these 21 solutions were extracted by liquid-liquid extraction with pentane-diethyl ether (2:1, 2 mL) and analysed by the method.

In contrast to section 7.1.1, duplicate analyses were not performed for every sample in sections 7.1.2-5 because of the large number of samples being analysed and the precision of the analytical method (as shown in Chapter 4). Some samples (approx. 10%) were selected at random and were analysed in duplicate as spot checks and all of these gave good precision (< 5% co-efficient of variance). In addition, duplicate preparation of all the extracts in 7.1.3, 7.1.4 and 7.1.5 accounted for any analytical variation, along with the much greater factor of sample variation.

7.1.3 The evolution of oak volatiles over time

Three oakwood samples from the preliminary trial (7.1.2 above) were chosen for further study. These were Troncais #2 (*Q. sessilis*), American #5 (*Q. alba*) and

American #3 medium grain (*Q. alba*). Further subsamples of these three oakwood planks were cut into two approximately equal portions and one piece of each wood was heated in a Carbolite constant temperature (+/- 1°C) oven fitted with a Eurotherm digital controller (Medos, Adelaide, Australia) at 200°C for 1 hour, giving six samples altogether (one heated and one unheated for each of the three woods). Each of the six samples was shaved into approx. 20 mm x 1 mm needles using a Skil 94H 400W rotary plane. For each treatment, the shavings were mixed thoroughly and subdivided into two approximately equal portions (masses of the shavings [prior to any heating] were: Troncais unheated #1 replicate (rep.) 32.96g, #2 rep. 33.87g; Troncais heated #1 rep. 17.41g, #2 rep. 20.01g; American unheated #1 rep. 30.41g, #2 rep. 29.24g; American heated #1 rep. 27.80g, #2 rep. 27.58g; American medium grain unheated #1 rep. 28.39g, #2 rep. 30.19g; American medium grain heated #1 rep. 34.67g, #2 rep. 36.50g) which were placed in Schott bottles (250 mL). Model wine (250 mL, 10% ethanol in water, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid) was added to each of the shavings. Two control samples were prepared by spiking model wine (250 mL) to obtain concentrations of 200 μg/L guaiacol and 4-methylguaiacol, and 500 μg/L 4-ethylphenol, 4-ethylguaiacol, *cis*-oak lactone, *trans*-oak lactone and vanillin. The 12 oak extracts and two controls were left to stand at room temperature. Subsamples (5 mL) were taken after 1, 2, 4 and 8 days and analysed by the method (12 assays at each time). After 8 days at room temp. (25°C) the model wine was separated from the shavings by decantation and filtration through glass wool. Each filtrate was subdivided into ampoules (5 mL in each, at least seven of each). Ampoules of the control solutions were similarly prepared. All the ampoules were sealed then stored at 50°C. Subsamples (5 mL) were analysed by the method (Chapter 4) after 10, 20, 40 and 80 days. An ampoule of each control and oak extract was analysed at each time.

7.1.4 The extent of oxidative formation of oak volatiles over time

Further subsamples of the same three oakwood planks (used in 7.1.3 above) were sawn in half. One half of each subsample was heated in a Carbolite constant

temperature (+/- 1°C) oven fitted with an Eurotherm digital controller (Medos, Adelaide, Australia) at 200°C for 1 hour. All six oak wood pieces were individually shaved using a Skil 94H 400W rotary plane into approx. 20 mm by 1 mm needles, mixed thoroughly and subdivided into two approximately equal portions (masses of the shavings [prior to any heating] were: Troncais unheated #1 replicate (rep.) 15.49g, #2 rep. 15.25g; Troncais heated #1 rep. 12.86g, #2 rep. 12.25g; American unheated #1 rep. 17.02g, #2 rep. 17.84g; American heated #1 rep. 23.63g, #2 rep. 21.31g; American medium grain unheated #1 rep. 25.23g, #2 rep. 21.42g; American medium grain heated #1 rep. 16.15g, #2 rep. 17.08g). The twelve samples of shavings were soaked in model wine (10% ethanol in water, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid) for 8 days at 25°C, after which the wood was removed by decantation and filtration and the 12 filtrates (5 mL aliquots) analysed by the method (as described in Chapter 4). Further aliquots (5 mL) were sealed into ampoules either under air or nitrogen, in duplicate for each, and analysed by the method after 40 days at 50°C (24 assays).

7.1.5 The formation of vanillin from coniferaldehyde

Four replicates of 250 mL model wine (10% ethanol in water, adjusted to pH 3.0 with potassium hydrogen tartrate and tartaric acid) were spiked to 1000 μg/L with coniferaldehyde (250 μL of 1.00 mg/mL solution in ethanol, 99% pure by GC/MS). Another 2 x 250 mL replicates of the same model wine were spiked to 1000 μg/L with vanillin (250 μL of 1.00 mg/mL solution in ethanol, 100% pure by GC/MS). Two of the coniferaldehyde replicates were spiked in an anaerobic fumehood, sealed and stored under nitrogen for the remainder of the trial. The other four solutions were spiked under normal aerobic conditions (on the bench in the lab), sealed and stored under air for the remainder of the trial. All six solutions were stored at 50°C with subsamples (5 mL) taken at zero, 10, 20 and 40 days and analysed by the method (as described in Chapter 4). All extractions of the anaerobic samples were done under anaerobic conditions. All extractions of the aerobic samples were done under normal aerobic conditions.

7.2 RESULTS AND DISCUSSION

7.2.1 Effects of air and temperature on the formation of guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone and vanillin in heated oak shavings

Effects due to the heating of oak are known (eg Spillman 1998, Wittowski *et al.* 1992) but the role of air in this heating is not known. Oak chips and innerstaves are sometimes toasted under an inert atmosphere, presumably due to a potential fire hazard. It is also possible that some ways of toasting barrels involve more exposure to air than others.

Shavings were made of a stave of French (Troncais, *Q. sessilis*) and a stave of American (*Q. alba*) oak and subjected to ultrasound under air (ie. approx. 20% oxygen) or argon (no oxygen) and then left at 25°C or heated with the same gas cover (ie. air or argon) at 175°C or 225°C for two hours. There was also a control set in which the shavings underwent no ultrasound or heat treatment. The shavings were soaked in model wine for 1 week then extracted and analysed.

The results are given in Table 7.1.

Table 7.1 Effects of air and temperature on the formation of oak volatiles

Oak	Atmosphere	Temp °C	Guaiacol µg/g (cv%)	4-MeG µg/g (cv%)	Vanillin µg/g (cv%)	*trans*-OL µg/g (cv%)	*cis*-OL µg/g (cv%)	OL Ratio *cis / trans*
French	Control	25	0.09 (32%)	0.01 (20%)	6.75 (28%)	7.79 (47%)	2.87 (21%)	0.4
French	Argon	25	0.09 (74%)	0.01 (73%)	7.30 (62%)	7.19 (74%)	3.01 (49%)	0.4
French	Air	25	0.07 (37%)	0.01 (27%)	6.55 (14%)	15.3 (20%)	2.82 (0%)	0.2
French	Argon	175	0.17 (38%)	0.16 (33%)	7.52 (28%)	10.7 (13%)	2.00 (11%)	0.2
French	Air	175	0.32 (13%)	0.33 (21%)	13.2 (1%)	11.1 (27%)	2.99 (14%)	0.3
French	Argon	225	4.88 (30%)	1.54 (25%)	10.2 (15%)	1.58 (101%)	0.74 (55%)	0.5
French	Air	225	10.0 (0%)	4.16 (7%)	22.5 (4%)	2.95 (37%)	3.02 (26%)	1.0
American	Control	25	0.03 (8%)	0.01 (16%)	2.78 (4%)	1.01 (4%)	17.9 (5%)	17.7
American	Argon	25	0.04 (6%)	0.01 (4%)	5.30 (2%)	1.70 (1%)	30.2 (1%)	17.8
American	Air	25	0.04 (48%)	0.01 (56%)	4.06 (51%)	1.30 (44%)	23.6 (46%)	18.1
American	Argon	175	0.09 (6%)	0.11 (19%)	4.22 (10%)	0.71 (11%)	9.80 (8%)	13.8
American	Air	175	0.21 (3%)	0.50 (37%)	22.5 (52%)	1.44 (2%)	20.0 (1%)	13.9
American	Argon	225	3.66 (7%)	1.41 (8%)	11.7 (2%)	0.54 (11%)	5.47 (8%)	10.1
American	Air	225	5.10 (20%)	2.07 (25%)	15.8 (16%)	0.83 (34%)	7.66 (25%)	9.2

Concentrations shown are in µg/g of unheated oak shavings and are the mean of four analyses – duplicate samples of shavings from the same stave were seperately treated and then extracted and analysed in duplicate. Temp = temperature, cv = coefficient of variance, 4-MeG = 4-methylguaiacol, *trans*-OL = *trans*-oak lactone, *cis*-OL = *cis*-oak lactone

The coefficients of variance were good (< 5%) between duplicate analyses, but not always as good between duplicate preparations (as can be seen in Table 7.1). In most cases, there is a considerable range in the level of variation, depending on the compound, eg. French oak heated in air at 225°C shows coefficients of variance that range from 0% (guaiacol) to 37% (*trans*-oak lactone). The heterogeneity of the wood is a possible source of variation, as despite thorough mixing of the shavings by hand prior to subdivision into the different treatments, the distribution obtained was not necessarily completely random.

Guaiacol and 4-methylguaiacol

Both the French and American oak samples showed similar trends for the generation of guaiacol and 4-methylguaiacol.

There was no effect due to ultrasound treatment of the oak shavings alone.

Heating the wood had a major influence. The concentrations of guaiacol and 4-methylguaiacol increased with increasing temperature.

Air was important, with twice as much guaiacol and 4-methylguaiacol formed in air, compared with argon, at both heating temperatures.

Guaiacol and 4-methylguaiacol are known to be formed by the pyrolysis of lignin (eg Wittowski *et al.* 1992, Spillman 1998). However, this is the first study to investigate the effect of the presence or absence of air on such generation.

Vanillin

The effect of heating in the presence or absence of air on the generation of vanillin was similar to the effect on guaiacol and 4-methylguaiacol. However, some differences were observed in the behaviour of the two oak samples. Both samples contained significant vanillin prior to any treatment. Ultrasonication had no effect on the French oak but resulted in a significant increase in vanillin concentration in the American oak.

In the French oak, heating to 175°C under argon made no significant difference to vanillin concentration, but the vanillin concentration doubled in the extracts heated in contact with air. At 225°C there was a ~30% increase in vanillin under argon and a ~400% increase with air.

Similarly, in the American oak, heating to 175°C under argon made no significant difference to vanillin concentration, but there was a ~500% increase with air. At 225°C, less vanillin was formed with air than at 175°C with only a ~400% increase. This is probably because decomposition is competing with generation and the rate of decomposition is higher with higher temperature.

Heating under argon at 225°C resulted in a ~300% increase.

These results indicate the importance of air to the formation of vanillin during heating.

Oak Lactone

The oak lactone isomers show far greater variation between extracts than any of the other compounds measured, indicating that their distribution throughout the wood is more heterogeneous. Oak lactone is an important constituent of oak prior to heating (Sefton *et al* 1993a).

There does not seem to be any significant, consistent formation of *cis*-oak lactone with heating the samples. Indeed, in the American oak, there was generally more *cis*-oak lactone present in the unheated shavings. This is in contrast to Maga (1989a & b) who stated that charring roughly tripled the amount of oak lactone present, although he did not state what constituted "charring" in his studies, nor the size of the oak pieces that he heated, nor whether the whole oak piece was analysed or just sections of the oak that were scraped. The oak shavings in this study were evenly toasted to a fairly dark brown at 175°C and a very dark brown at 225°C but did not blacken, burn or char. It is likely that Maga's observation is based on wood that did burn in some parts, and the increase is due to pyrolysis at these localised portions of the surface of the oak wood, in contrast to the even controlled heating used in this trial.

The coefficients of variance were always greater for *trans*-oak lactone, typically twice as great as for *cis*-oak lactone, and even higher for the French oak in air at 25°C.

It is hard to assess the effect of heating due to the large variation in oak lactone concentrations. Nevertheless there is usually less oak lactone present in the wood

heated to 225°C than that heated to 175°C, possibly due to degradation or volatilisation of both isomers with increased temperature. However, there is more oak lactone in the samples heated in air, than those heated in argon, at both heating temperatures. This suggests that a relatively small formation effect, such as the oxidation of lipids, is competing with the degradation.

The ratio of the *cis*- to *trans*- oak lactone isomers was reasonably consistent for the American oak extracts, but unusual ratios were observed in the French oak extracts. Table 7.1 shows that the ratio change was mostly due to variation in the amount of *trans*-oak lactone produced.

Summary

Air was important in the formation of guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone and vanillin with the heating of these fine oak shavings.
Experiments are in progress (outside of this thesis) to determine the relationship of the size of the oak pieces on the formation of volatiles with and without air at different heating temperatures.

7.2.2 Evolution of volatile compounds by extraction from oak shavings and formation in solution

This experiment was conducted to determine whether oak volatiles are derived from oak solely by extraction, or whether such volatiles can also be generated from involatile precursor forms by hydrolysis and / or oxidation. Were such generation from precursors to be possible, then additional oak flavour could develop by generation during bottle ageing. The possibility that this process may take place has been suggested by the steady linear increase observed in guaiacol and vanillin concentration (after the initial relatively rapid asymptotic extraction over the first 6 weeks [guaiacol] and 32 weeks [vanillin]) in accumulation profiles for volatile compounds during barrel ageing of wines (Spillman *et al.* 1997 and 1998b).

Shavings were made of 21 oak wood samples, from different regions in France and America, toasted, and extracted in relatively acidic (pH 3.0) model wine for a week at 25°C and analysed by the method. In order to determine whether additional quantities of guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone and vanillin could be formed in model wine oak extracts after the shavings were removed, the extracts were heated in the presence of air to 80°C for 10 days and then analysed by the method. The concentrations measured are shown in Tables IVa & b in Appendix IV.

The ratio of *cis* / *trans* isomers found in the American oak (*Q. alba*) samples are within the typical range of samples analysed in our laboratory in conjunction with industry trials and in agreement with Waterhouse and Towey 1994, Guichard *et al.* 1995, Towey and Waterhouse 1996a&b, Swan 1997, Maga 1996, Spillman 1998 and Sefton *et al.* 1993a.

In the chestnut oak sample (*Q. prinus*), however, there was about twice as much *trans*-oak lactone as *cis*-oak lactone. This finding has not been previously reported and is in contrast with the findings of Swan 1997 who reports ratios of cis / *trans* from 2 to 1 up to 4 to 1 in chestnut oaks. Furthermore, in none of the oak wood species analysed by Waterhouse and Towey 1996a&b was the ratio of *cis* / *trans* less than or equal to one, and for all their American wood samples (except the Oregon oak *Q. garryana*) the ratios were within the range of 5 to 8 *cis* to 1 *trans*. Similarly, Maga 1996 does not report more *trans* than *cis* in any example, except when citing earlier literature where the isomers were incorrectly assigned (as discussed in section 1.3.4.2).

The ratio of *cis* / *trans* isomers found in the French oak samples are not all typical – Limousin #3, Troncais #3, Troncais #5 and Vosges #3, all had more *trans*-oak lactone than *cis*-oak lactone. This has never previously been observed in the literature (eg. Spillman 1998, Sefton *et al.* 1993a, Maga 1996, Towey and Waterhouse 1996a&b, Waterhouse and Towey 1994). Furthermore, other pieces of the same oak, initially seasoned in the same countries, did not show these ratios (Spillman 1998, Sefton *et al.* 1993a). This could possibly be because the previous analytical methods were not as

accurate as that developed in the thesis, however it is unlikely that the relative amounts of *cis*- and *trans*-oak lactone would vary by so much, even if their absolute values were inaccurate. More likely, these results differ because the oakwood pieces had been cellared for over ten years between the analyses reported previously (Sefton *et al.* 1993a) and the results in this thesis. Indeed Sefton (*et al.* 1993a) and Maga (1989a) state that seasoning can influence the *cis / trans* ratio. This change in the ratio of oak lactone isomers with storage time could be of interest for further research, now that accurate analytical tools are available to measure *cis*- and *trans*-oak lactone.

There were higher concentrations of all the oak volatiles in the extracts stored at 80°C for 10 days. Compared to the results from the 25°C extracts every compound increased in every extract, except for *trans*-oak lactone in American #6, which had the same low concentration (0.004 μg/g wood). Over all 21 oak extracts, the average increase (inc.), co-efficient of variance (cv) and range, for each compound, were:

guaiacol	238% inc.	16% cv	range 181% - 300%;
4-methylguaiacol	156% inc.	9% cv	range 140% - 179%;
trans-oak lactone	138% inc.	16% cv	range 100% - 183%;
cis-oak lactone	152% inc.	19% cv	range 132% - 243%;
vanillin	177% inc.	8% cv	range 151% - 206%.

Not all volatiles increased to the same extent, both within the same oak extract and over all 21 oakwoods analysed.

Inspection of the *cis* to *trans* ratios of oak lactone isomers at 25°C and after ten days at 80°C, shows that the ratio for each oak extract has not significantly changed for those samples containing more than trace amounts (0.03 μg/g) of each isomer. This further supports the conclusion reached in section 4.2.1.2, ie. that significant *cis / trans* isomerisation does not occur in the weakly acidic medium of wines during ageing in the barrel, or storage in the bottle, as has been suggested elsewhere (Chatonnet 1991, Waterhouse and Towey 1994, Piggott *et al.* 1995).

These preliminary results encouraged the following more detailed study.

7.2.3 The evolution of oak volatiles over time

Three oak staves were chosen from the 21 investigated in the preliminary experiment in order to study the rate at which oak volatiles were extracted into wine, and to investigate the increase in the concentrations of oak volatiles over time after the oak was removed from the wine. Thus further subsamples of these three oakwood planks were cut into two, and one half of each subsample was toasted at 200°C for 1 hour, giving six samples altogether, heated and unheated for each of the three woods. The extraction of oak compounds from the shavings at room temperature was monitored over an eight day period, then the oak was removed and the filtrates were stored at 50°C and the concentrations of guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone and vanillin were monitored over 80 days. The experiments were carried out at a typical wine pH of 3.4 and without any inert gas protection. The heating at 50°C was intended to simulate the effect of ageing for a much longer time period at room temperature (ie the ampoules represent bottles of wine, made in contact with oak, then removed from the oak and cellared for several years). These conditions are less extreme than those used in the preliminary trial (7.2.2). The results are shown in Table 7.2.

As the concentrations of all the analytes in all the controls are consistently near the initial spike values it can be concluded that no problems with the analysis occurred at any of the sampling times (eg. oxidative loss, or absorption of the volatiles into the plastic lids did not occur to any significant degree).

In order to verify that there was no artefactual generation of the volatiles of interest (eg guaiacol) as a result of the analyses, SPME analyses were done on all the controls and on one duplicate of each oak shaving extract, heated and unheated, for the day 40 samples (data not shown). There was no significant difference between the concentrations of the analytes by SPME as compared to the liquid-liquid extracts. No significant generation of guaiacol, 4-methylguaiacol, *cis*- or *trans*-oak lactone or vanillin was observed in any sample as a result of the analyses involving liquid-liquid extracts.

Table 7.2 Study on the Evolution of Oak Volatiles - by Compound

Guaiacol - Model Wine Oak Extracts

Identity	RT Day 0 ug/L	RT Day 1 ug/L	RT Day 2 ug/L	RT Day 4 ug/L	RT Day 8 = 50C day 0 ug/L	50C Day 10 ug/L	50C Day 10 % inc (+/-)	50C Day 20 ug/L	50C Day 20 % inc (+/-)	50C Day 40 ug/L	50C Day 40 % inc (+/-)	50C Day 80 ug/L	50C Day 80 % inc (+/-)
Control	207	205	200	207	211	200		199		205		203	
Troncais, Unheated	0	1	1	2	2	7		8		8		9	
American, Unheated	0	3	3	3	4	5		5		7		10	
American Med Grain Unheated	0	40	40	43	50	51	2% (0%)	51	2% (0%)	52	4% (0%)	52	4% (2%)
Troncais, Heated	0	72	74	83	87	89	2% (2%)	92	6% (5%)	101	16% (11%)	114	31% (19%)
American, Heated	0	122	127	144	146	156	7% (6%)	158	8% (5%)	167	14% (0%)	176	21% (0%)
American Med Grain Heated	0	66	66	89	110	112	2% (2%)	112	2% (2%)	114	4% (1%)	119	8% (0%)

4-Methylguaiacol - Model Wine Oak Extracts

Identity	RT Day 0 ug/L	RT Day 1 ug/L	RT Day 2 ug/L	RT Day 4 ug/L	RT Day 8 = 50C day 0 ug/L	50C Day 10 ug/L	50C Day 10 % inc (+/-)	50C Day 20 ug/L	50C Day 20 % inc (+/-)	50C Day 40 ug/L	50C Day 40 % inc (+/-)	50C Day 80 ug/L	50C Day 80 % inc (+/-)
Control	200	201	197	200	199	204		199		199		198	
Troncais, Unheated	0	trace	trace	trace	trace	trace		trace		trace		trace	
American, Unheated	0	trace	trace	trace	trace	trace		trace		ND		ND	
American Med Grain Unheated	0	2	2	2	2	2		1		1		ND	
Troncais, Heated	0	79	88	94	94	99	5% (3%)	99	5% (2%)	100	6% (1%)	100	6% (1%)
American, Heated	0	45	46	49	47	54	15% (5%)	59	26% (6%)	53	13% (13%)	53	13% (13%)
American Med Grain Heated	0	17	19	23	20	23	15% (0%)	27	35% (0%)	21	5% (0%)	23	15% (0%)

trans-Oak Lactone - Model Wine Oak Extracts

Identity	RT Day 0 ug/L	RT Day 1 ug/L	RT Day 2 ug/L	RT Day 4 ug/L	RT Day 8 = 50C day 0 ug/L	50C Day 10 ug/L	50C Day 10 % inc (+/-)	50C Day 20 ug/L	50C Day 20 % inc (+/-)	50C Day 40 ug/L	50C Day 40 % inc (+/-)	50C Day 80 ug/L	50C Day 80 % inc (+/-)
Control	504	501	500	505	506	512		505		512		503	
Troncais, Unheated	0	4	4	5	5	5		5		5		5	
American, Unheated	0	trace	trace	trace	trace	trace		trace		trace		trace	
American Med Grain Unheated	0	456	458	487	502	607	21% (3%)	591	18% (3%)	582	16% (5%)	571	14% (3%)
Troncais, Heated	0	8	9	10	10	10		10		10		10	
American, Heated	0	trace	trace	trace	trace	trace		trace		trace		trace	
American Med Grain Heated	0	295	302	362	395	452	14% (2%)	447	13% (2%)	442	12% (1%)	436	10% (2%)

cis-Oak Lactone - Model Wine Oak Extracts

Identity	RT Day 0 ug/L	RT Day 1 ug/L	RT Day 2 ug/L	RT Day 4 ug/L	RT Day 8 = 50C day 0 ug/L	50C Day 10 ug/L	50C Day 10 % inc (+/-)	50C Day 20 ug/L	50C Day 20 % inc (+/-)	50C Day 40 ug/L	50C Day 40 % inc (+/-)	50C Day 80 ug/L	50C Day 80 % inc (+/-)
Control	497	498	476	497	500	500		504		500		493	
Troncais, Unheated	0	17	17	19	20	20	0% (0%)	21	5% (5%)	21	5% (5%)	22	10% (0%)
American, Unheated	0	trace	trace	trace	trace	trace		trace		trace		trace	
American Med Grain Unheated	0	2200	2210	2700	2670	3270	22% (0%)	3300	24% (2%)	3370	26% (0%)	3310	24% (0%)
Troncais, Heated	0	27	28	29	30	30	0% (0%)	30	0% (0%)	30	0% (0%)	31	3% (0%)
American, Heated	0	trace	trace	trace	trace	trace		trace		trace		trace	
American Med Grain Heated	0	1950	2010	2210	2460	2870	17% (5%)	3000	22% (5%)	3120	27% (5%)	2990	22% (6%)

Vanillin - Model Wine Oak Extracts

Identity	RT Day 0 ug/L	RT Day 1 ug/L	RT Day 2 ug/L	RT Day 4 ug/L	RT Day 8 = 50C day 0 ug/L	50C Day 10 ug/L	50C Day 10 % inc (+/-)	50C Day 20 ug/L	50C Day 20 % inc (+/-)	50C Day 40 ug/L	50C Day 40 % inc (+/-)	50C Day 80 ug/L	50C Day 80 % inc (+/-)
Control	501	496	496	514	511	517		538		506		542	
Troncais, Unheated	0	653	666	820	862	1040	21% (4%)	1070	24% (2%)	1080	25% (0%)	1240	44% (1%)
American, Unheated	0	390	428	514	515	703	37% (9%)	719	40% (8%)	731	42% (8%)	866	68% (4%)
American Med Grain Unheated	0	359	371	433	437	583	33% (1%)	682	56% (0%)	697	59% (2%)	890	104% (14%)
Troncais, Heated	0	2920	3150	3260	3410	5070	49% (6%)	5910	73% (0%)	6240	83% (2%)	6830	100% (2%)
American, Heated	0	1975	2010	2060	2320	3380	46% (6%)	3960	71% (9%)	4560	97% (10%)	5550	139% (13%)
American Med Grain Heated	0	1330	1340	1680	1790	2590	45% (2%)	2980	66% (4%)	3250	82% (1%)	4080	128% (10%)

All concentrations are in ug/L model wine, adjusted to assume 25g shavings in 250mL model wine and are the mean from two duplicate preparations.
RT = Room temperature (25C), Med = Medium
"% inc" refers to the mean percentage increase in the concentration of the analyte after the stated storage time at 50C as compared to the RT day 8 = 50C day 0 sample.
The bracketed "(+/-)" values show the deviation of each duplicate from the mean, ie (0%) means both preparations increased by the same amount (to the nearest percent).
The percentage increases are not shown for the controls or for samples containing less than or equal to 10 ug/L.

The extraction of the oak volatiles into the model wine was rapid. After one day at room temperature, most analytes were 70-100% of the concentration they were after eight days at room temperature.

Guaiacol

The evolution of guaiacol was never complete in the room temperature samples, similar to the evolution observed by Spillman *et al.* (1998b).
Evolution at 50°C was relatively slow, except where the amounts of guaiacol were low (< 5 μg/L) prior to storage at 50°C. After 80 days at this temperature, the guaiacol concentration was still increasing slowly – its evolution still incomplete.
Thus, the evolution of guaiacol at room temperature is not due to the hydrolysis of *soluble* precursors.

4-Methylguaiacol

Unlike guaiacol, the evolution of 4-methylguaiacol at room temperature was complete after two to four days. After eight days, when the shavings were removed and the filtrate stored at 50°C, there was more 4-methylguaiacol released from precursors (up to 20 days). The mean amount of 4-methylguaiacol generated over this time was an average of 5 μg/L (94 to 99 μg/L) in the heated Troncais oak extract, 15 μg/L (47 to 59 μg/L) for heated American oak seasoned in Australia, and 7 μg/L (20 μg/L to 27 μg/L) for the medium grain American oak. After this time, however, only 1 μg/L of additional 4-methylguaiacol was generated over the next 60 days for the heated Troncais, and the two heated American oaks showed significant decreases down to mean values of 53 μg/L and 23 μg/L (medium grain) after 80 days. Obviously, the 4-methylguaiacol is being converted to other products. Whether this is a competitive process occurring at the same time as the generation or whether the generation stops and degradation starts is unclear from the data.

Oak Lactone

The Troncais oak contained low amounts of both oak lactones. There was no change in the amount of *trans*-oak lactone extracted from the Troncais oak with extended storage at 50°C. Similarly there were only very small increases (1 – 2 μg/L) in *cis*-oak lactone concentration over 80 days at 50°C.

One American oak showed only trace amounts of both oak lactones throughout, however the medium grain American oak had by far the highest oak lactone content (about 100 times more than the French oak). At 25°C, the evolution of *trans*-oak lactone was incomplete, even after eight days. This was also the case for *cis*-oak lactone for the heated oak. However, the accumulation of *cis*-oak lactone reached equilibrium after four days for the unheated oak. There were big increases in the concentration of both oak lactone isomers in the first ten days at 50°C but little change thereafter. Thus, the formation of oak lactone from soluble precursors is probably hydrolytic. The initial increase for the *trans*-oak lactone was greater for the toasted oak, however the initial increase for the *cis*-oak lactone was greater for the untoasted oak – although these observations are based on only one sample of oakwood. More oak samples with significant oak lactone concentration need to be investigated. Experiments are in progress (outside of this thesis).

Vanillin

Vanillin showed higher concentrations than all the other compounds analysed in most samples. In similar behaviour to guaiacol, vanillin was extracted out more slowly than the other volatiles, and continued to increase in concentration the longer the wines were stored. However the increase for vanillin was much more substantial - after the shavings were removed, the vanillin concentration still increased steadily to up to approx. 2.4 times the concentration after 80 days at 50°C, with the biggest rate of increase occurring in the first 10 days. After 80 days, the evolution of vanillin was still incomplete. There was about four to six times as much vanillin in the heated shavings as the unheated.

7.2.4 The extent of oxidative formation of oak volatiles over time

The previous experiments have determined that some flavour compounds are formed from the hydrolysis of precursors over time. To investigate whether oxygen contributed to this effect, subsamples of the same three pieces of oakwood were extracted and analysed as for the previous trial, except that the extracts were initially stored under nitrogen for eight days at room temperature prior to the first set of analyses and then the filtrates were subdivided into two sets of ampoules – one set was sealed under nitrogen, the other under air. The second set of analyses was done after 40 days storage at 50°C. Results are compared to the midpoint of the previous trial and can be seen in table 7.3. Care should be taken in the comparison of the results from this experiment with those of the previous study (section 7.2.3) as although the shavings were made from the same staves, the wood samples are not necessarily homogeneous, and the average size of shavings may have varied between preparations.

The increase in guaiacol was relatively small (up to 8%) after 40 days storage at 50°C. No air effect was observed, except for the heated *Q. alba* medium grain which had an extra 17% increase for the extracts stored under air.

Very little 4-methylguaiacol was detected in the unheated oak shavings (less than or equal to 2 μg/L). Among the heated oak samples, small increases (up to 8%) over the storage period, and also due to air, were observed for the Troncais oak and the medium grain American oak. No differences at all were observed for the other American oak. 4-Methylguaiacol increased and then decreased over time in the main trial under air - it cannot be seen from the one sampling viewpoint (at 40 days) in this second trial if there was also an increase and then a decrease under nitrogen.

Table 7.3 Evolution of oak volatiles with and without air

Oak Identity	N_2 Guaiacol day zero ug/L	N_2 Guaiacol day 40 ug/L	N_2 Guaiacol day 40 % inc (+/-)	Air Guaiacol day 40 ug/L	Air Guaiacol day 40 % inc (+/-)	N_2 4-MeG day zero ug/L	N_2 4-MeG day 40 ug/L	N_2 4-MeG day 40 % inc (+/-)	Air 4-MeG day 40 ug/L	Air 4-MeG day 40 % inc (+/-)
Troncais, Unheated	2	4		4		< 1	< 1		< 1	
American, Unheated	4	4		4		< 1	< 1		< 1	
Q Alba Med Grain Unheated	51	55	8% (3%)	53	4% (2%)	2	2		2	
Troncais, Heated	84	89	6% (0%)	89	6% (2%)	101	107	6% (0%)	109	8% (6%)
American, Heated	72	78	8% (0%)	77	7% (1%)	30	30	0% (0%)	30	0% (0%)
Q Alba Med Grain Heated	75	81	8% (0%)	94	25% (2%)	24	24	0% (0%)	26	8% (2%)

Oak Identity	N_2 trans-OL day zero ug/L	N_2 trans-OL day 40 ug/L	N_2 trans-OL day 40 % inc (+/-)	Air trans-OL day 40 ug/L	Air trans-OL day 40 % inc (+/-)	N_2 cis-OL day zero ug/L	N_2 cis-OL day 40 ug/L	N_2 cis-OL day 40 % inc (+/-)	Air cis-OL day 40 ug/L	Air cis-OL day 40 % inc (+/-)
Troncais, Unheated	4	4		4		21	26	24% (1%)	26	24% (1%)
American, Unheated	3	3		3		14	19	36% (5%)	19	36% (5%)
Q Alba Med Grain Unheated	488	548	12% (7%)	537	10% (0%)	2980	3370	13% (5%)	3290	10% (0%)
Troncais, Heated	4	4		4		19	26	37% (4%)	26	37% (4%)
American, Heated	< 1	< 1		< 1		4	7		7	
Q Alba Med Grain Heated	356	378	6% (1%)	445	25% (1%)	2260	2510	11% (1%)	2910	29% (1%)

Oak Identity	N_2 Vanillin day zero ug/L	N_2 Vanillin day 40 ug/L	N_2 Vanillin day 40 % inc (+/-)	Air Vanillin day 40 ug/L	Air Vanillin day 40 % inc (+/-)
Troncais, Unheated	860	1000	16% (1%)	1110	29% (2%)
American, Unheated	465	562	21% (2%)	611	31% (1%)
Q Alba Med Grain Unheated	447	621	39% (2%)	732	64% (2%)
Troncais, Heated	3450	3880	12% (0%)	4340	26% (3%)
American, Heated	1710	1890	11% (1%)	2270	64% (2%)
Q Alba Med Grain Heated	1770	2120	20% (4%)	3260	84% (5%)

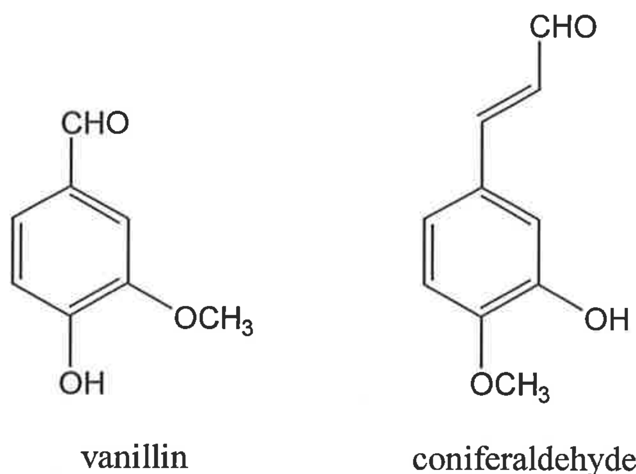
All concentrations are in ug/L model wine, adjusted to assume 10g shavings in 100mL model wine.
Mean value from two duplicate preparations shown.
"% inc" refers to the mean percentage increase in the concentration of the analyte after the 40 days storage after the oak shavings were removed
The bracketed "(+/-)" values show the deviation of each duplicate from the mean, ie (0%) means both preparations increased by the same amount (to the nearest percent).
The percentage increases are not shown for concentrations less than 10 ug/L.
Model wine was 10% ethanol in water, pH 3.4 (adjusted with potassium hydrogen tartrate and tartaric acid).
4-MeG = 4-methylguaiacol, trans-OL = *trans*-oak lactone, cis-OL = *cis*-oak lactone
Air = stored under air
N_2 = stored under nitrogen

Only the medium grain American oak had significant quantities (> 30 μg/L) of total oak lactone. Moderate increases in both isomers (6-25% for *trans* and 11-37% for *cis*) were observed over the 40 days storage at 50°C, presumably from reactions involving soluble precursors. An air effect was observed only for the toasted shavings of this oak, with an extra 18-19% of both isomers being generated during storage under air. This implies that more oak lactone precursors were formed during the toasting of this oak, which then reacted to form more *cis*- and *trans*-oak lactone, presumably oxidatively, during storage. The approximate *cis* : *trans* ratios were 6.1 : 1 for the unheated oak and 6.5 : 1 for the same oak toasted. This difference is probably due to more *trans*-oak lactone being volatilised during the toasting than *cis*-oak lactone, since *trans* is the more volatile of the isomers.

Vanillin showed much larger increases (up to almost double the original concentration) than any other analyte over the 40 days storage at 50°C. Air had a major effect on the extent of vanillin generation, which is in agreement with the observations of Maarse and van den Berg (1989) who concluded that more vanillin accumulated in oak extracts that were stored in contact with oxygen. After the 40 days, the levels of vanillin were about 10-50% higher in the samples stored in the presence of air, as compared to those stored under nitrogen for the same period. The larger differences were observed for the American oaks, which initially had about half the level of vanillin than that found in the Troncais oak. For each oak extract, the percentage increase in vanillin for aerobic storage versus anaerobic storage was larger than the increase for any other of the compounds analysed.

The generation of volatiles observed in the main trial (section 7.2.3) occurred under aerobic conditions. The results from the trial in this section show that generation also occurs anaerobically after the oak shavings are removed. In most cases where an increase was observed under air, a similar (but usually smaller increase) was observed under nitrogen. This implies that the generation effects observed in the main trial are still very relevant, however the magnitude of generation has been enhanced by the presence of air, to varying degrees, depending on the analyte. This effect is small (< 20%) for all volatiles, except vanillin, in which the values could be enhanced by 10-50%.

7.2.5 The formation of vanillin from coniferaldehyde



Puech (1981), Reazin (1981) and Puech *et al.* (1984) proposed a model in which vanillin can be formed from coniferaldehyde (a major component of toasted oak) in the presence of oxygen, presumably by oxidative cleavage of the double bond. This model has seldom been questioned in the literature, and never investigated experimentally. Since vanillin can also be formed from coniferaldehyde by a reverse Aldol condensation, a reaction that does not require oxygen, it is possible that this formation could occur anaerobically in the acidic medium of wine. To test these hypotheses analytically, duplicate extracts of separate model wine solutions of coniferaldehyde (1000 μg/L) and vanillin (1000 μg/L) were stored under air at 50°C. Subsamples (5 mL) were analysed for vanillin by the method immediately and after 10, 20 and 40 days storage. Several ampoules of both the original coniferaldehyde solutions were sealed under nitrogen and stored at 50°C. Vanillin concentration in an ampoule of each of these solutions was also measured by the method at each sampling time. The results are shown in Table 7.4.

Table 7.4 Vanillin concentration in μg/L

	Zero	Nitrogen 10 days @ 50C	Nitrogen 20 days @ 50C	Nitrogen 40 days @ 50C
Conif Soln#1	ND	18	26	26
Conif Soln#2	ND	18	24	26

	Zero	Air 10 days @ 50C	Air 20 days @ 50C	Air 40 days @ 50C
Conif Soln#1	ND	35	35	22
Conif Soln#2	ND	36	34	20
Vanillin Soln#1	1000	1010	1010	993
Vanillin Soln#2	1000	954	1050	1000

Conif means Coniferaldehyde. ND means not detected above 1 μg/L.

No vanillin was formed at time zero.

In the anaerobic coniferaldehyde solutions, there was a small amount of vanillin formed hydrolytically. After 20 days at 50°C this formation appears to have reached equilibrium, with approximately the same level of vanillin present as in the 40 day samples. From this data, it is not clear whether the concentrations remained the same or whether vanillin continued to accumulate after 20 days but then degraded back to the same level after 40 days. The total vanillin content formed under anaerobic conditions was only 2.6% of the original coniferaldehyde concentration.

After the first 10 days storage at 50°C, twice as much vanillin was present in the coniferaldehyde spiked model wines stored under air, than in those stored under nitrogen. Approximately the same concentrations were observed in the aerobically stored samples after 20 days. However, it is not clear from this data whether the concentrations remained the same or whether vanillin continued to accumulate after 10 days but then degraded back to the same level after 20 days. The highest value shows a vanillin concentration of only 3.6% of the original coniferaldehyde concentration.

Over 40 days aerobic storage at 50°C there was no significant degradation in the vanillin solutions, yet approx. 40% of the vanillin was lost from the equivalent coniferaldehyde solutions between days 20 and 40. The reasons for this are unknown.

Coniferaldehyde is not a significant contributor to the formation of vanillin in contrast to what was previously thought (eg Puech 1981, Reazin 1981, Puech *et al.* 1984, Maarse and van den Berg 1989).

7.2.6 The accumulation of vanillin in wine aged in oak barrels

Spillman (1998) studied the accumulation of oak volatiles in red, white and model wines aged in oak barrels. Spillman's analytical method (Spillman 1998, Sefton *et al.* 1993a&b) used exhaustive continuous liquid-liquid extraction of at least 500 mL of wine with three successive aliquots of Freon F11 and required a concentration step. Although this gave adequate results for other oak volatiles, the vanillin analysis was poor (as discussed in Chapter 4.2.1). The analyses of vanillin in the samples studied by Spillman (1998) were repeated by the author of this thesis, with technical assistance from Dimitra Capone, by the method developed in Chapter 4. The accumulation of vanillin in model wine aged in oak barrels over a 93 week period is shown in Figure 7.1 (Figure 8.3 in Spillman 1998).

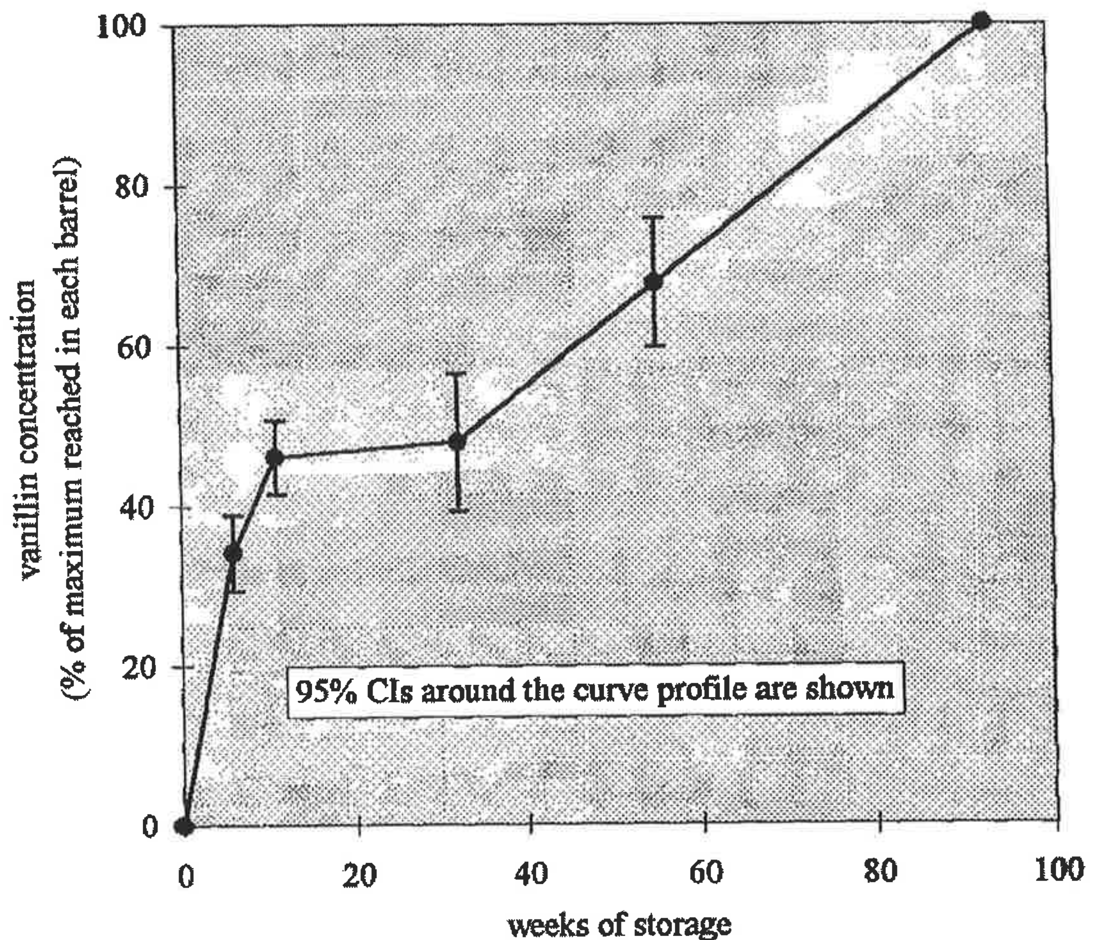


Figure 7.1 **The accumulation of vanillin in model wine stored in four American and four Limousin oak barrels**

The curve shows an asymptotic increase of vanillin at first, then the curve is relatively flat at about week 32, and then the rate of vanillin accumulation increases again at a linear rate, doubling in concentration between weeks 32 and 93. The rapid early increase is presumably mainly due to extraction from the wood and possibly also from hydrolysis of precursors. This is followed by little or no increase, showing that the generation of vanillin by anaerobic hydrolysis during this period was minimal. Sulfur dioxide addition ceased after the first six months. Therefore after the sulfur dioxide was consumed, oxidative conditions prevailed and vanillin was accumulated at a linear rate due to oxidation, possibly coupled with hydrolysis. Maarse and van den Berg (1989) also concluded that more vanillin accumulated in oak extracts that were stored in contact with oxygen.

7.3 Summary

Wines made in contact with oak can continue to develop oak-derived aroma and flavour with bottle age as flavour compounds are formed from the hydrolysis of precursors over time. The presence of air can sometimes enhance the amount of each volatile generated, with the largest increases observed for vanillin. This is not very relevant to commercial wines, which are usually protected from oxidation by blanketing with inert gas, addition of antioxidants and fermentation over yeast lees. However, experimental model wine oak extracts, such as those described in this chapter, can be susceptible to such an effect. Brandies aged in oak casks are not protected from air, and do show increased concentrations of vanillin, especially in older barrels (eg Puech 1981, Puech and Moutounet 1992). Coniferaldehyde has only a very small (< 4%) contribution to the formation of vanillin.

8 Summary

Oak products, be they in the form of cork-bark closures, barrels or chips, affect wine aroma and flavour.

New methodology was developed in this thesis that enabled the author to accurately and precisely quantitate TCA, the prime cause of 'cork taint' and other chloroanisoles in large numbers of wine and cork samples. The method has been used in many problem-solving trials for industry, has had relevant research applications (eg. Chapter 5, Pollnitz *et al.* 1996, Howland *et al.* 1997, Howland 1997, Liacopoulos *et al.* 1999, Capone *et al.* 1999, Barker *et al.* 2000), and is offered as a commercial service to industry (http://winetitles.com.au/awri/tca.html).

New methodology was developed in this thesis for the rapid, precise and accurate analysis of oak volatiles. Unlike all previously published research, these methods were optimised to minimise the generation of artefacts, from oak derived precursors, occurring as a result of the analyses. This new methodology made it possible to analyse oak volatiles in large numbers of wine and oak samples in conjunction with industry trials and is offered as a commercial service to industry (http://winetitles.com.au/awri/oak.html). Furthermore, now that this analytical method is available, many areas of relevant oak and wine research are opened up (eg. Chapters 6 and 7, Spillman *et al.* 1997, Osicka 1997, Spillman *et al.* 1998a, Spillman 1998, Pollnitz *et al.* 1999, Pollnitz *et al.* 2000a-c).

Appendix I (page 1 of 9)

Oak volatiles extracted from oak shavings under different extraction conditions
All concentrations are in µg/g of oak shavings prior to heating

Q. Alba **fine grain A – Unheated shavings (118.75 g / 1 L model wine) – Conc µg/g**

				Inj. 200°C			Inj. 250°C		
	SPME1	**SPME2**	**SPME3**	**2:1 #1**	**2:1 #2**	**2:1 #3**	**2:1 #1**	**2:1 #2**	**2:1 #3**
Guaiacol	0.06	0.07	0.05	0.07	0.06	0.08	**0.13**	**0.15**	**0.16**
4-MeG	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	ND	ND	ND	ND	ND	ND	ND	ND	ND
trans OL	2.01	1.98	1.99	1.99	1.96	1.95	2.03	1.97	2.01
cis vs 101	*13.6*	*13.5*	*13.0*	*13.5*	*13.1*	*13.2*	*13.7*	*13.5*	*13.4*
cis vs 90	14.1	14.1	14.2	13.9	13.6	13.8	14.3	13.9	14.0
Vanillin	18	10	16	5.77	5.72	5.79	6.00	5.73	5.84

Q. Alba **fine grain B – Unheated shavings (125.55 g / 1 L model wine) – Conc µg/g**

	SPME			pent. Inj 200°C			pent. Inj 225°C			pent. Inj 250°C		
	#1	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**
Guaiacol	0.21	0.20	0.20	0.21	0.21	0.21	0.20	0.21	0.21	0.19	0.21	0.22
4-MeG	0.32	0.32	0.32	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.32
4-EP	0.33	0.34	0.33	0.33	0.31	0.34	0.32	0.32	0.32	0.30	0.33	0.33
4-EG	1.12	1.24	1.18	2.95	2.83	3.03	2.78	2.68	2.79	2.68	2.83	2.74
trans OL	6.80	6.79	6.70	6.62	6.61	6.65	6.61	6.74	6.65	6.59	6.65	6.62
cis vs 101	*67.0*	*64.8*	*65.9*	*65.9*	*66.7*	*65.9*	*64.4*	*66.6*	*66.3*	*65.7*	*66.1*	*66.4*
cis vs 90	86.4	83.5	84.4	85.2	85.2	86.0	83.6	87.6	86.0	82.8	85.2	86.0
Vanillin	4	2	2	2.00	2.17	2.05	1.90	1.90	1.91	1.76	1.88	2.16

Q. Alba **fine grain B – Unheated shavings (125.55 g / 1 L model wine) – Conc µg/g**

	SPME			2:1 p:e Inj 200°C			2:1 p:e Inj 225°C			2:1 p:e Inj 250°C		
	#1	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**
Guaiacol	0.21	0.20	0.20	**0.23**	**0.23**	**0.24**	**0.23**	**0.24**	**0.25**	**0.25**	**0.26**	**0.26**
4-MeG	0.32	0.32	0.32	0.32	0.31	0.32	0.32	0.32	0.32	0.32	0.32	0.31
4-EP	0.33	0.34	0.33	0.32	0.32	0.31	0.32	0.32	0.32	0.32	0.32	0.32
4-EG	1.12	1.24	1.18	0.80	0.79	0.80	0.74	0.75	0.74	0.77	0.76	0.75
trans OL	6.80	6.79	6.70	6.56	6.52	6.54	6.66	6.56	6.56	6.63	6.54	6.58
cis vs 101	*67.0*	*64.8*	*65.9*	*66.3*	*65.9*	*66.2*	*67.5*	*67.1*	*66.9*	***68.4***	***68.0***	***68.3***
cis vs 90	86.4	83.5	84.4	86.0	87.6	86.8	87.6	87.6	87.6	**90.0**	**90.0**	**90.0**
Vanillin	4	2	2	1.52	1.55	1.54	1.65	1.61	1.57	1.89	1.55	1.60

Abbreviations etc. are explained on the last page of the table (page 9).

Appendix I (page 2 of 9)
Oak volatiles extracted from oak shavings under different extraction conditions
All concentrations are in μg/g of oak shavings prior to heating

Q. Alba **fine grain B – Unheated shavings (125.55 g / 1 L model wine) – Conc μg/g**

	SPME			ether Inj 200°C			ether Inj 225°C			ether Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.21	0.20	0.20	**0.26**	**0.30**	**0.32**	**0.57**	**0.58**	**0.60**	**1.58**	**1.55**	**1.53**
4-MeG	0.32	0.32	0.32	0.33	0.33	0.32	**0.33**	**0.33**	**0.33**	**0.34**	**0.34**	**0.34**
4-EP	0.33	0.34	0.33	0.36	0.33	0.32	0.31	0.31	0.31	0.31	0.32	0.31
4-EG	1.12	1.24	1.18	0.74	0.74	0.74	0.68	0.68	0.69	0.71	0.71	0.70
trans OL	6.80	6.79	6.70	6.47	6.57	6.51	6.59	6.67	6.61	6.67	6.73	6.63
cis vs 101	*67.0*	*64.8*	*65.9*	*63.5*	*65.3*	*65.2*	*66.8*	*67.7*	*67.9*	***70.3***	***72.1***	***70.8***
cis vs 90	86.4	83.5	84.4	82.8	84.4	85.2	**89.2**	**90.8**	**90.0**	**93.2**	**95.6**	**94.8**
Vanillin	4	2	2	1.89	1.66	1.56	1.55	1.54	1.53	1.59	1.59	1.59

Q. Alba **medium grain A – Unheated shavings (132.14 g / 1 L model wine) - (μg/g)**

				Inj. 200°C			Inj. 250°C		
	SPME1	SPME2	SPME3	2:1 #1	2:1 #2	2:1 #3	2:1 #1	2:1 #2	2:1 #3
Guaiacol	0.07	0.06	0.06	0.06	0.06	0.06	**0.13**	**0.28**	**0.11**
4-MeG	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	ND	ND	ND	ND	ND	ND	ND	ND	ND
trans OL	3.93	3.97	3.96	3.92	3.92	3.93	3.91	3.92	4.01
cis vs 101	14.4	14.5	14.3	14.2	14.2	14.2	14.2	14.3	14.5
cis vs 90	*15.3*	*15.4*	*15.2*	*15.1*	*15.1*	*15.1*	*15.1*	*15.2*	*15.4*
Vanillin	10	10	6	4.50	4.53	4.56	4.59	4.59	4.70

Q. Alba **medium grain B – Unheated shavings (72.73 g / 1 L model wine) – (μg/g)**

				Inj. 200°C			Inj. 250°C		
	SPME1	SPME2	SPME3	2:1 #1	2:1 #2	2:1 #3	2:1 #1	2:1 #2	2:1 #3
Guaiacol	0.07	0.04	0.05	0.04	0.04	0.04	**0.07**	**0.11**	**0.11**
4-MeG	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	ND	ND	ND	ND	ND	ND	ND	ND	ND
trans OL	1.66	1.76	1.70	1.66	1.66	1.66	1.66	1.66	1.68
cis vs 101	22.3	21.4	21.6	21.6	21.7	21.7	22.0	22.0	22.0
cis vs 90	*23.1*	*22.8*	*22.5*	*22.3*	*22.4*	*22.3*	*22.7*	*23.1*	*22.7*
Vanillin	19	19	23	5.94	5.75	5.87	**6.10**	**6.39**	**6.23**

Abbreviations etc. are explained on the last page of the table (page 9).

Appendix I (page 3 of 9)

Oak volatiles extracted from oak shavings under different extraction conditions

All concentrations are in μg/g of oak shavings prior to heating

Chestnut Oak A – Unheated shavings (141.24 g / 1 L model wine) – Conc in μg/g

	SPME			pent. Inj 200°C			pent. Inj 225°C			pent. Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.79	0.77	0.78	0.81	0.79	0.73	0.79	0.80	0.81	0.80	0.75	0.81
4-MeG	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.36	0.38	0.39	0.87	1.00	0.96	0.86	0.89	0.86	0.89	0.89	0.87
trans OL	37.0	36.7	36.6	38.2	37.2	37.5	37.9	37.4	37.9	37.9	37.1	37.9
cis vs 101	18.3	18.2	18.3	18.5	18.1	18.2	18.5	18.2	18.3	18.7	18.2	18.5
cis vs 90	*19.9*	*19.5*	*19.5*	*19.7*	*19.3*	*19.5*	*19.8*	*19.7*	*19.8*	*19.5*	*18.8*	*19.9*
Vanillin	4	4	3	3.40	3.26	3.12	3.52	3.46	3.03	3.48	3.48	3.43

Chestnut Oak A – Unheated shavings (141.24 g / 1 L model wine) – Conc in μg/g

	SPME			2:1 p:e Inj 200°C			2:1 p:e Inj 225°C			2:1 p:e Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.79	0.77	0.78	**0.81**	**0.81**	**0.81**	**0.87**	**0.84**	**0.83**	**0.86**	**0.97**	**0.85**
4-MeG	0.02	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.02
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.36	0.38	0.39	0.25	0.26	0.25	0.24	0.24	0.24	0.26	0.25	0.25
trans OL	37.0	36.7	36.6	36.5	36.3	36.5	36.3	36.6	37.0	36.0	36.5	36.7
cis vs 101	18.3	18.2	18.3	17.9	18.1	18.1	18.0	18.1	18.1	18.0	18.3	18.4
cis vs 90	*19.9*	*19.5*	*19.5*	*19.1*	*19.2*	*19.2*	*19.3*	*19.3*	*19.3*	*19.3*	*19.6*	*19.5*
Vanillin	4	4	3	3.51	3.38	3.41	3.48	3.48	3.46	3.32	3.42	3.35

Chestnut Oak A – Unheated shavings (141.24 g / 1 L model wine) – Conc in μg/g

	SPME			ether Inj 200°C			ether Inj 225°C			ether Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.79	0.77	0.78	**0.84**	**0.82**	**0.84**	**1.02**	**1.06**	**1.07**	**1.70**	**1.77**	**1.83**
4-MeG	0.02	0.03	0.02	0.03	0.03	0.03	**0.04**	**0.04**	**0.04**	**0.04**	**0.05**	**0.05**
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.36	0.38	0.39	0.24	0.25	0.24	0.23	0.23	0.22	0.23	0.23	0.22
trans OL	37.0	36.7	36.6	35.9	36.0	36.0	36.3	36.2	35.8	36.3	36.2	36.3
cis vs 101	18.3	18.2	18.3	17.8	17.6	17.7	17.9	17.8	18.0	**18.8**	**18.7**	**18.8**
cis vs 90	*19.9*	*19.5*	*19.5*	*18.8*	*19.0*	*18.8*	*19.4*	*19.1*	*19.2*	***20.0***	***20.5***	***20.3***
Vanillin	4	4	3	3.45	3.38	3.35	3.41	3.37	3.29	3.40	3.41	3.40

Abbreviations etc. are explained on the last page of the table (page 9).

Appendix I (page 4 of 9)
Oak volatiles extracted from oak shavings under different extraction conditions
All concentrations are in μg/g of oak shavings prior to heating

Chestnut Oak B – Unheated shavings (101.99 g / 1 L model wine) – Conc in μg/g

	SPME			**pent. Inj 200°C**			**pent. Inj 225°C**			**pent. Inj 250°C**		
	#1	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**
Guaiacol	0.76	0.74	0.77	0.81	0.78	0.76	0.78	0.78	0.75	0.79	0.77	0.73
4-MeG	0.04	0.05	0.05	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.37	0.37	0.36	0.87	0.85	0.82	0.87	0.85	0.87	0.85	0.87	0.84
trans OL	40.8	41.6	41.6	41.8	41.4	41.2	41.6	42.0	40.7	41.7	41.4	40.8
cis vs 101	18.3	19.5	18.5	18.6	18.7	18.2	18.5	18.8	18.3	18.7	18.3	18.1
cis vs 90	19.1	19.9	19.0	19.6	19.5	19.2	19.4	19.5	19.1	19.3	18.8	18.6
Vanillin	3	3	5	2.61	2.65	2.64	2.66	2.65	2.61	2.86	2.72	2.56

Chestnut Oak B – Unheated shavings (101.99 g / 1 L model wine) – Conc in μg/g

	SPME			**2:1 p:e Inj 200°C**			**2:1 p:e Inj 225°C**			**2:1 p:e Inj 250°C**		
	#1	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**
Guaiacol	0.76	0.74	0.77	**0.80**	**0.82**	**0.81**	**0.81**	**0.79**	**0.82**	**0.88**	**0.85**	**0.82**
4-MeG	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.37	0.37	0.36	0.23	0.26	0.24	0.22	0.23	0.22	0.23	0.23	0.23
trans OL	40.8	41.6	41.6	40.2	40.3	40.3	40.7	40.5	40.6	40.3	40.3	40.2
cis vs 101	18.3	19.5	18.5	18.1	18.0	18.0	18.4	18.4	18.2	18.3	18.5	18.3
cis vs 90	*19.1*	*19.9*	*19.0*	*19.1*	*19.1*	*19.2*	*19.2*	*19.4*	*18.9*	*19.3*	*19.5*	*19.2*
Vanillin	3	3	5	2.76	2.76	2.76	**2.84**	**2.90**	**2.83**	**2.86**	**2.83**	**2.89**

Chestnut Oak B – Unheated shavings (101.99 g / 1 L model wine) – Conc in μg/g

	SPME			**ether Inj 200°C**			**ether Inj 225°C**			**ether Inj 250°C**		
	#1	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**
Guaiacol	0.76	0.74	0.77	**0.85**	**0.83**	**0.89**	**1.13**	**1.07**	**1.13**	**1.77**	**1.79**	**1.83**
4-MeG	0.04	0.05	0.05	0.05	0.05	0.05	**0.06**	**0.06**	**0.07**	**0.07**	**0.07**	**0.08**
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.00
4-EG	0.37	0.37	0.36	0.22	0.22	0.22	0.20	0.21	0.21	0.22	0.22	0.22
trans OL	40.8	41.6	41.6	40.0	39.9	39.4	39.9	40.0	39.7	40.2	40.1	39.9
cis vs 101	18.3	19.5	18.5	18.1	18.1	18.0	18.2	18.0	18.2	18.6	18.7	18.9
cis vs 90	*19.1*	*19.9*	*19.0*	*18.9*	*18.7*	*18.8*	*19.0*	*19.0*	*19.0*	***19.4***	***19.8***	***19.6***
Vanillin	3	3	5	2.85	2.74	2.75	2.73	2.73	2.72	2.79	2.77	2.78

Abbreviations etc. are explained on the last page of the table (page 9).

Appendix I (page 5 of 9)
Oak volatiles extracted from oak shavings under different extraction conditions
All concentrations are in μg/g of oak shavings prior to heating

Q. Alba **fine grain A – Heated shavings (87.18 g / 1 L model wine) – Conc in μg/g**

				Inj. 200°C			Inj. 250°C		
	SPME1	SPME2	SPME3	2:1 #1	2:1 #2	2:1 #3	2:1 #1	2:1 #2	2:1 #3
Guaiacol	0.09	0.09	0.09	0.10	0.10	0.09	**0.17**	**0.14**	**0.12**
4-MeG	0.21	0.21	0.21	0.20	0.20	0.20	0.22	0.21	0.20
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	ND	ND	ND	ND	ND	ND	ND	ND	ND
trans OL	1.65	1.72	1.65	1.67	1.63	1.59	1.72	1.67	1.63
cis vs 101	11.1	11.2	11.1	10.8	11.0	10.7	11.3	10.9	10.7
cis vs 90	11.5	11.5	11.5	11.1	11.1	10.8	11.5	11.3	10.9
Vanillin	7	5	6	11.8	11.5	11.6	11.1	11.6	11.6

Q. Alba **fine grain B – Heated shavings (125.01 g / 1 L model wine) – Conc μg/g**

	SPME			pent. Inj 200°C			pent. Inj 225°C			pent. Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.30	0.31	0.31	0.32	0.30	0.32	0.30	0.30	0.31	**0.33**	**0.33**	**0.33**
4-MeG	0.34	0.35	0.34	0.33	0.34	0.34	0.33	0.33	0.33	0.34	0.33	0.33
4-EP	0.28	0.31	0.29	0.31	0.31	0.32	0.29	0.31	0.31	0.28	0.29	0.30
4-EG	1.07	1.11	1.10	2.65	2.57	2.95	2.55	2.57	2.72	2.68	2.58	2.80
trans OL	5.28	5.46	5.36	5.18	5.27	5.30	5.10	5.29	5.33	5.18	5.30	5.34
cis vs 101	57.4	60.2	58.6	56.4	57.8	58.4	57.1	57.8	58.7	57.3	59.0	58.9
cis vs 90	*72.3*	*75.4*	*74.1*	*72.6*	*72.8*	*73.1*	*72.7*	*73.1*	*73.3*	*72.8*	*73.9*	*73.8*
Vanillin	3	4	4	4.56	5.76	5.79	5.05	6.62	6.25	5.63	6.85	6.50

Q. Alba **fine grain B – Heated shavings (125.01 g / 1 L model wine) – Conc μg/g**

	SPME			2:1 p:e Inj 200°C			2:1 p:e Inj 225°C			2:1 p:e Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.30	0.31	0.31	0.32	0.33	0.33	0.32	0.34	0.35	0.30	0.33	0.34
4-MeG	0.34	0.35	0.34	0.34	0.34	0.34	0.34	0.33	0.33	0.33	0.34	0.34
4-EP	0.28	0.31	0.29	0.28	0.28	0.27	0.28	0.28	0.27	0.27	0.27	0.26
4-EG	1.07	1.11	1.10	0.74	0.73	0.74	0.75	0.74	0.74	0.75	0.75	0.73
trans OL	5.28	5.46	5.36	5.26	5.35	5.28	5.26	5.29	5.23	5.28	5.35	5.28
cis vs 101	57.4	60.2	58.6	57.2	57.6	57.3	57.7	59.0	58.0	58.2	59.0	58.9
cis vs 90	*72.3*	*75.4*	*74.1*	*72.5*	*73.3*	*72.8*	*73.3*	*73.4*	*73.4*	*73.4*	*73.7*	*73.8*
Vanillin	3	4	4	8.32	8.32	8.08	8.48	8.16	8.24	8.48	8.24	8.40

Abbreviations etc. are explained on the last page of the table (page 9).

Appendix I (page 6 of 9)

Oak volatiles extracted from oak shavings under different extraction conditions

All concentrations are in µg/g of oak shavings prior to heating

Q. Alba **fine grain B – Heated shavings (125.01 g / 1 L model wine) – Conc µg/g**

	SPME			ether Inj 200°C			ether Inj 225°C			ether Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.30	0.31	0.31	**0.39**	**0.44**	**0.44**	**0.99**	**0.98**	**0.93**	**2.22**	**2.27**	**2.35**
4-MeG	0.34	0.35	0.34	0.33	0.33	0.34	0.34	0.34	0.34	0.35	0.35	0.36
4-EP	0.28	0.31	0.29	0.28	0.28	0.28	0.27	0.27	0.27	0.27	0.28	0.28
4-EG	1.07	1.11	1.10	0.65	0.65	0.65	0.60	0.60	0.61	0.63	0.62	0.63
trans OL	5.28	5.46	5.36	5.15	5.14	5.25	5.18	5.13	5.29	5.30	5.26	5.40
cis vs 101	57.4	60.2	58.6	56.5	57.0	57.5	57.1	57.1	58.3	58.3	58.4	59.5
cis vs 90	*72.3*	*75.4*	*74.1*	*72.4*	*72.2*	*72.3*	*72.4*	*72.7*	*73.8*	*73.7*	*74.6*	*75.9*
Vanillin	3	4	4	8.16	8.08	8.40	8.16	8.16	8.32	8.40	8.32	8.56

Q. Alba **medium grain A – Heated shavings (105.31 g / 1 L model wine) – (µg/g)**

				Inj. 200°C			Inj. 250°C		
	SPME1	**SPME2**	**SPME3**	**2:1 #1**	**2:1 #2**	**2:1 #3**	**2:1 #1**	**2:1 #2**	**2:1 #3**
Guaiacol	0.10	0.10	0.10	**0.11**	**0.11**	**0.11**	**0.14**	**0.14**	**0.13**
4-MeG	0.27	0.27	0.27	0.27	0.26	0.26	0.27	0.26	0.26
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	ND	ND	ND	ND	ND	ND	ND	ND	ND
trans OL	3.91	3.94	3.99	3.98	3.95	3.84	3.98	4.01	3.82
cis vs 101	14.5	14.7	14.8	14.4	14.3	14.1	14.7	14.5	14.1
cis vs 90	15.0	15.2	15.2	15.0	14.7	14.5	15.1	15.0	14.5
Vanillin	17.9	18.1	18.9	21.5	21.7	21.9	21.6	20.2	20.9

Q. Alba **medium grain B – Heated shavings (120.30 g / 1 L model wine) – (µg/g)**

				Inj. 200°C			Inj. 250°C		
	SPME1	**SPME2**	**SPME3**	**2:1 #1**	**2:1 #2**	**2:1 #3**	**2:1 #1**	**2:1 #2**	**2:1 #3**
Guaiacol	0.08	0.08	0.08	**0.09**	**0.09**	**0.10**	**0.10**	**0.11**	**0.10**
4-MeG	0.36	0.35	0.35	0.35	0.36	0.36	0.34	0.35	0.35
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	ND	ND	ND	ND	ND	ND	ND	ND	ND
trans OL	1.52	1.47	1.44	1.43	1.49	1.50	1.42	1.50	1.48
cis vs 101	17.4	16.8	16.6	16.5	17.0	17.2	16.5	17.2	17.2
cis vs 90	*18.3*	*18.3*	*18.3*	*17.4*	*18.0*	*18.3*	*17.6*	*18.4*	*18.1*
Vanillin	8.5	7.5	8.4	20.1	20.5	20.4	20.0	20.0	20.3

Abbreviations etc. are explained on the last page of the table (page 9).

Appendix I (page 7 of 9)
Oak volatiles extracted from oak shavings under different extraction conditions
All concentrations are in μg/g of oak shavings prior to heating

Chestnut Oak A – Heated shavings (45.94 g / 1 L model wine) – Conc in μg/g

	SPME			pent. - Inj 200°C			pent. - Inj 225°C			pent. - Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.52	0.54	0.53	0.54	0.55	0.54	1.52	0.61	0.53	1.31	0.92	0.55
4-MeG	1.01	1.00	1.00	0.96	1.00	0.99	1.03	1.02	1.00	1.04	1.06	0.99
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.22	0.22	0.21	0.52	0.51	0.52	0.45	0.55	0.45	0.46	0.51	0.51
trans OL	44.2	45.1	44.4	44.2	44.4	43.5	44.2	44.4	43.5	44.8	45.1	44.0
cis vs 101	21.5	21.5	21.3	21.2	21.3	21.2	21.4	21.4	20.9	22.0	22.2	21.4
cis vs 90	21.8	21.7	21.4	21.7	21.8	21.3	22.2	21.6	21.1	22.0	21.7	21.7
Vanillin	56	59	62	60.5	56.4	61.6	58.1	58.8	62.7	63.6	59.4	63.3

Chestnut Oak A – Heated shavings (45.94 g / 1 L model wine) – Conc in μg/g

	SPME			2:1 p:e Inj 200°C			2:1 p:e Inj 225°C			2:1 p:e Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.52	0.54	0.53	**0.55**	**0.55**	**0.56**	**0.72**	**0.58**	**0.64**	**0.78**	**0.77**	**0.75**
4-MeG	1.01	1.00	1.00	1.02	1.01	1.03	1.01	1.04	0.99	1.01	1.05	1.00
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.22	0.22	0.21	0.15	0.14	0.14	0.13	0.14	0.14	0.11	0.08	0.09
trans OL	44.2	45.1	44.4	43.8	43.8	43.8	44.2	44.2	44.2	44.0	44.4	44.2
cis vs 101	21.5	21.5	21.3	21.1	21.1	21.0	21.5	21.5	21.5	21.6	21.6	21.7
cis vs 90	21.8	21.7	21.4	21.6	21.6	21.5	21.6	21.7	21.7	21.6	22.0	22.2
Vanillin	56	59	62	65.1	66.2	66.6	63.8	67.5	62.9	62.3	66.2	69.4

Chestnut Oak A – Heated shavings (45.94 g / 1 L model wine) – Conc in μg/g

	SPME			ether - Inj 200°C			ether - Inj 225°C			ether - Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.52	0.54	0.53	**1.28**	**1.18**	**1.06**	**2.53**	**2.51**	**2.39**	**5.12**	**6.86**	**5.70**
4-MeG	1.01	1.00	1.00	1.01	1.02	1.02	**1.03**	**1.03**	**1.03**	**1.03**	**1.06**	**1.04**
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.22	0.22	0.21	0.14	0.14	0.13	0.14	0.13	0.13	0.13	0.14	0.14
trans OL	44.2	45.1	44.4	42.4	42.4	42.7	43.1	43.1	43.1	43.1	43.1	43.3
cis vs 101	21.5	21.5	21.3	20.8	20.7	20.7	21.1	21.2	21.1	**21.8**	**22.0**	**21.8**
cis vs 90	21.8	21.7	21.4	21.1	20.7	20.9	21.3	21.8	21.8	**22.2**	**22.4**	**22.4**
Vanillin	56	59	62	62.0	61.6	61.6	61.6	62.3	61.6	61.4	63.3	62.7

Abbreviations etc. are explained on the last page of the table (page 9).

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Oak volatiles extracted from oak shavings under different extraction conditions

All concentrations are in μg/g of oak shavings prior to heating

Chestnut Oak B – Heated shavings (77.51 g / 1 L model wine) – Conc in μg/g

	SPME			pent. - Inj 200°C			pent. - Inj 225°C			pent. - Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.39	0.38	0.40	**0.53**	**0.53**	**0.46**	**0.77**	**0.63**	**0.50**	**0.94**	**0.69**	**0.50**
4-MeG	0.49	0.51	0.51	0.50	0.50	0.48	0.52	0.49	0.49	0.50	0.49	0.50
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.17	0.17	0.15	0.34	0.35	0.32	0.37	0.36	0.38	0.35	0.34	0.37
trans OL	35.7	36.0	36.3	35.6	35.5	34.8	35.7	35.5	35.0	35.9	35.6	34.8
cis vs 101	16.1	15.7	15.9	15.6	15.5	15.5	15.7	15.6	15.5	15.9	15.7	15.5
cis vs 90	16.5	16.3	16.1	15.9	15.7	15.5	16.0	15.6	15.5	16.5	16.0	15.5
Vanillin	15.5	16.8	17.0	25.8	24.9	25.0	26.6	25.8	24.5	28.5	25.2	27.1

Chestnut Oak B – Heated shavings (77.51 g / 1 L model wine) – Conc in μg/g

	SPME			2:1 p:e Inj 200°C			2:1 p:e Inj 225°C			2:1 p:e Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.39	0.38	0.40	0.38	0.40	0.41	**0.41**	**0.46**	**0.45**	**0.45**	**0.57**	**0.58**
4-MeG	0.49	0.51	0.51	0.52	0.53	0.52	0.52	0.51	0.51	0.51	0.53	0.51
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.17	0.17	0.15	0.10	0.10	0.10	0.10	0.10	0.10	0.08	0.08	0.08
trans OL	35.7	36.0	36.3	35.0	35.0	34.7	34.8	35.2	34.7	35.2	35.2	35.0
cis vs 101	16.1	15.7	15.9	15.2	15.4	15.1	15.5	15.6	15.4	15.6	15.7	15.7
cis vs 90	16.5	16.3	16.1	15.6	15.9	15.6	15.7	15.9	15.7	15.9	16.3	15.7
Vanillin	15.5	16.8	17.0	25.2	25.3	24.9	25.3	26.7	26.1	26.4	26.6	26.2

Chestnut Oak B – Heated shavings (77.51 g / 1 L model wine) – Conc in μg/g

	SPME			ether - Inj 200°C			ether - Inj 225°C			ether - Inj 250°C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Guaiacol	0.39	0.38	0.40	**0.65**	**0.76**	**0.67**	**1.14**	**1.39**	**1.23**	**3.24**	**3.12**	**3.15**
4-MeG	0.49	0.51	0.51	0.50	0.51	0.50	0.50	0.51	0.51	**0.53**	**0.55**	**0.53**
4-EP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-EG	0.17	0.17	0.15	0.11	0.11	0.10	0.10	0.10	0.10	0.10	0.11	0.11
trans OL	35.7	36.0	36.3	33.9	34.6	33.7	35.4	35.6	35.1	35.6	35.7	35.1
cis vs 101	16.1	15.7	15.9	15.5	15.5	15.5	15.5	15.6	15.5	15.7	15.9	15.5
cis vs 90	16.5	16.3	16.1	15.5	15.6	15.5	15.6	16.0	15.6	*16.4*	*16.8*	*16.3*
Vanillin	15.5	16.8	17.0	24.8	24.8	24.4	24.4	24.5	24.0	25.0	25.0	24.6

Abbreviations etc. are explained on the last page of the table (page 9).

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All analyses were done on 5 mL replicate aliquots of the model wine oak extract.
#1, #2, #3 refer to the specific replicate

SPME means solid phase micro-extraction
pent. means pentane, 2:1 p:e means 2:1 pentane : ether (liquid/liquid extraction)
Inj. means injector block temperature.
ND means not detected above 0.01 μg/g (approx. equivalent to 1 μg/L model wine)
4-MeG means 4-methylguaiacol
4-EP means 4-ethylphenol
4-EG means 4-ethylguaiacol
trans OL means *trans*-oak lactone
cis vs 101 means *cis*-oak lactone as quantitated versus the *m/z* 101 ion of d_4- *cis*-oak lactone
cis vs 90 means *cis*-oak lactone as quantitated versus the *m/z* 90 ion of d_4- *cis*-oak lactone

All SPME analyses for vanillin are shaded to show they are inaccurate – this is due to factors including the relatively lower volatility of vanillin or the relatively poor affinity of vanillin for the SPME fibre giving it low signal to noise ratio. Pentane extracts of vanillin were also poor in sensitivity, but were still considerably better than the sensitivity obtained from SPME of vanillin.

Bold concentration values in the table represent those values which show a significant difference in the concentration of the analyte measured by liquid-liquid extraction versus SPME, or for vanillin concentrations which show a significant increase at a higher liquid injection temperature versus liquid injection at 200°C. These differences are summarised in tables 4.5 to 4.8.

Italicised values are used for concentrations of *cis*-oak lactone vs *m/z* 90 that are considerably higher than their *cis*-oak lactone vs *m/z* 101 counterparts, due to a co-eluting ion with the d_4-*cis*-oak lactone 101 ion. There were no other significant co-eluter effects with the quantitation ions of any other analyte or deuterated standard.

4-Ethylguaiacol differences were not deemed to be significant, as the analysis of the model wines spiked with unlabelled reference standards only (data in Table 4.4) showed that these differences were due to differences in solubility and volatility of 4-ethylguaiacol as compared to it's internal standard, d_4-4-ethylphenol, which was not a strict isotopically labelled analogue.

Appendix II Investigation into deuterium exchange of d_4-4-ethylphenol during gas chromatography

The ratios of the ions, *m/z* 126, 125, 124, 123, 122 of d_4-4-ethylphenol (internal standard) remained the same before and after extraction; thus there was no back exchange into the wine during sample preparation. Indeed, during the synthesis (Scheme 2.5), strong acid and high temperature for a prolonged period was required for exchange ortho to the phenol group, and the other hydrogens were not exchanged at all under these conditions. Separate solutions (diluted to *ca* 0.5 mg/mL in dichloromethane) of d_4-4-ethylphenol and unlabelled 4-ethylphenol were analysed at the start, middle and end of a 200 run sequence of wine extracts. They were injected with the injector block temperature set at 200°C, 220°C and 250°C on each occasion, otherwise the GC conditions were as for the method, as described in Chapter 4. The ions monitored were *m/z* 118, 119, 120, 121, 122 for 4-ethylphenol and *m/z* 122, 123, 124, 125 and 126 for d_4-4-ethylphenol.

For all the analyses, there was no significant difference in the ratios of the ions monitored for either compound at all injection temperatures. Thus, no significant deuterium exchange occurred during sample preparation, injection or chromatography.

Appendix III

Mean concentrations of guaiacol, 4-methylguaiacol (4-MeG), 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG), *trans*-oak lactone (OL), *cis*-oak lactone (OL) and vanillin measured in the same red wine aged in new French and American oak barrels from different cooperages

Oak source and supplier #	Guaiacol µg/L (cv%)	4-MeG µg/L (cv%)	4-EP µg/L (cv%)	4-EG µg/L (cv%)	*trans*-OL µg/L (cv%)	*cis*-OL µg/L (cv%)	Vanillin µg/L (cv%)
American Oak #1	27 (15%)	10 (34%)	496 (9%)	36 (3%)	64 (39%)	601 (1%)	559 (24%)
American Oak #2	38 (4%)	14 (4%)	527 (2%)	33 (2%)	52 (0%)	473 (1%)	562 (1%)
Fine Grain American #2	30 (3%)	14 (4%)	519 (8%)	37 (4%)	82 (1%)	713 (16%)	622 (2%)
American Oak #3	22 (12%)	9 (53%)	680 (13%)	45 (14%)	63 (1%)	795 (4%)	452 (33%)
American Oak #4	19 (6%)	3 (11%)	564 (10%)	34 (6%)	83 (9%)	714 (2%)	291 (13%)
American Oak #5	36 (35%)	10 (42%)	552 (55%)	32 (47%)	51 (3%)	525 (7%)	342 (13%)
American Oak #6	50 (3%)	15 (4%)	566 (28%)	32 (18%)	57 (0%)	452 (0%)	640 (1%)
American Oak #7	40 (0%)	14 (16%)	432 (1%)	29 (2%)	56 (9%)	487 (28%)	770 (4%)
Fine Grain French #1	26 (9%)	16 (7%)	460 (2%)	31 (5%)	271 (62%)	345 (0%)	507 (8%)
Fine Grain French #2	40 (0%)	23 (6%)	385 (4%)	26 (4%)	115 (79%)	159 (47%)	465 (10%)
Medium Grain French #2	19 (9%)	8 (32%)	564 (18%)	34 (16%)	170 (17%)	188 (12%)	288 (13%)
Fine Grain French #6	25 (10%)	11 (3%)	511 (4%)	29 (2%)	114 (35%)	187 (4%)	483 (2%)
Medium Grain French #6	24 (18%)	10 (36%)	434 (4%)	28 (3%)	122 (11%)	189 (31%)	421 (5%)
Med/Fine Gr. French #8	29 (8%)	13 (9%)	646 (15%)	37 (20%)	117 (35%)	261 (4%)	445 (8%)
Fine Grain French #9	29 (7%)	17 (11%)	453 (0%)	28 (0%)	91 (71%)	129 (43%)	495 (5%)
Fine/Med Gr. French #9	24 (1%)	16 (1%)	390 (5%)	28 (5%)	92 (14%)	131 (17%)	479 (2%)
Fine Grain French #10	22 (24%)	10 (63%)	569 (7%)	33 (9%)	87 (8%)	191 (29%)	381 (28%)
French Oak #11	22 (na)	11 (na)	474 (na)	31 (na)	234 (na)	333 (na)	370 (na)
French Oak #12	20 (10%)	8 (68%)	523 (6%)	31 (2%)	173 (8%)	226 (10%)	295 (30%)

cv = co-efficient of variance, na = not applicable. Wine from two barrels of each type were analysed, except for French Oak #11 which was only one barrel.
#1, #2 etc. are arbitrary numbers assigned to each unique supplier (eg. American Oak #1 and Fine Grain French #1 are from the same cooperage)

Appendix IV

Table IVa - Oak volatiles in model wine oak extracts after one week at 25°C

Oak Identity	**Mass (g)**	**Guaiacol (µg/g)**	**4-MeG (µg/g)**	**Vanillin (µg/g)**	***trans*-OL (µg/g)**	***cis*-OL (µg/g)**	***cis / trans* ratio**
American #1	6.68	0.874	1.21	42.5	0.55	4.49	8.2
Amer. #2 (Fine Gr.)	6.38	0.555	1.07	62.7	0.56	5.45	9.7
Amer. #3 (Med. Gr.)	5.99	0.583	1.10	36.4	1.89	9.36	5.0
American #4	5.52	0.604	1.48	35.3	0.01	0.01	1.0
American #5	4.31	0.677	1.16	41.2	0.01	0.02	2.0
American #6	8.27	0.471	0.67	22.2	0.004	0.01	2.5
Chestnut (Amer.)	5.61	0.495	0.90	67.2	8.26	4.31	**0.5**
Limousin #1	6.03	0.463	2.12	63.2	0.03	0.18	6.9
Limousin #2	9.85	0.325	1.03	24.0	1.09	4.80	4.4
Limousin #3	8.84	0.314	1.75	36.2	0.08	0.05	**0.6**
Limousin #4	7.64	0.427	1.47	44.3	3.41	9.23	2.7
Troncais #1	4.50	0.603	1.52	55.8	0.32	1.04	3.2
Troncais #2	7.39	0.420	1.13	57.5	0.55	4.72	8.6
Troncais #3	7.88	0.383	1.07	26.9	4.10	3.44	**0.8**
Troncais #4	9.37	0.387	1.29	45.7	0.01	0.06	4.0
Troncais #5	4.55	0.488	1.43	57.4	2.37	0.20	**0.1**
Vosges #1	7.08	0.479	1.79	61.7	0.04	0.20	5.2
Vosges #2	9.15	0.370	1.43	31.7	0.06	0.08	1.2
Vosges #3	8.63	0.318	1.25	34.6	7.50	1.45	**0.2**
Vosges #4	9.26	0.446	1.52	54.7	0.02	0.05	2.0
Vosges #5	6.11	0.547	1.17	51.2	1.55	7.62	4.9

All concentrations are in µg/g of unheated oak shavings. Amer. = America.
Med. = Medium. Gr = grain. 4MeG = 4-methylguaiacol. OL = oak lactone.

Table IVb - Oak volatiles in model wine oak extracts after ten days at 80°C

Oak Identity	Mass (g)	Guaiacol (μg/g)	4-MeG (μg/g)	Vanillin (μg/g)	*trans*-OL (μg/g)	*cis*-OL (μg/g)	*cis / trans* ratio
American #1	6.68	1.58	1.75	70.8	0.72	6.11	8.5
Amer. #2 (Fine. Gr.)	6.38	1.32	1.58	109	0.75	7.47	10.0
Amer. #3 (Med. Gr.)	5.99	1.24	1.77	65.1	2.79	14.3	5.1
American #4	5.52	1.12	2.08	55.5	0.01	0.02	2.0
American #5	4.31	1.43	1.94	76.5	0.03	0.04	1.3
American #6	8.27	0.89	1.06	41.3	0.004	0.01	2.5
Chestnut (Amer.)	5.61	1.46	1.52	129	12.0	6.55	**0.5**
Limousin #1	6.03	1.39	3.55	112	0.03	0.27	8.4
Limousin #2	9.85	0.64	1.44	39.3	1.41	6.20	4.4
Limousin #3	8.84	0.73	2.70	63.2	0.09	0.07	**0.8**
Limousin #4	7.64	0.99	2.16	75.8	4.45	12.2	2.7
Troncais #1	4.50	1.71	2.66	108	0.55	1.97	3.6
Troncais #2	7.39	1.04	1.54	91.5	0.69	6.31	9.1
Troncais #3	7.88	0.85	1.70	46.7	6.08	5.27	**0.9**
Troncais #4	9.37	1.06	2.13	87.3	0.02	0.09	4.5
Troncais #5	4.55	1.35	2.45	110.5	3.80	0.31	**0.1**
Vosges #1	7.08	1.21	2.71	102	0.05	0.30	6.0
Vosges #2	9.15	0.93	2.41	61.7	0.09	0.11	1.2
Vosges #3	8.63	0.92	2.23	71.2	12.0	2.43	**0.2**
Vosges #4	9.26	0.96	1.99	82.6	0.03	0.06	2.0
Vosges #5	6.11	1.16	1.73	81.9	2.06	10.5	5.1

All concentrations are in μg/g of unheated oak shavings. Amer. = America.
Med. = Medium. Gr = grain. 4MeG = 4-methylguaiacol. OL = oak lactone.

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