# Influence of vineyard posts type on the chemical and sensorial composition of Sauvignon blanc and Merlot noir wines

by

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# Declaration

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# Summary

In recent years South African wines have been under the spotlight due to references in the international wine media to a distinctive ‘burnt rubber’ character. Many winemakers and wine experts argued that the peculiar character could be ascribed to winemaking errors linked to mismanaged fermentation. An alternative possible source of the taint was identified in the coal tar creosote used as a wood preservative in vineyard trellis systems. South African regulations allow for the use of creosoted utility poles in agricultural land, but in Europe and USA their usage has been highly regulated and even banned for certain applications. Contamination of foodstuff by polycyclic aromatic hydrocarbons (PAHs) is one of the major motives for the banning of creosote in Europe and USA. Some of the compounds in the creosote mixture display very strong odour characteristics and for this reason it became the focus of attention for the present study.

The overall aim of this study was to determine if vines trellised with creosoted posts could accumulate or absorb the various malodorous compounds deriving from the wood treatment onto the grape berries. These compounds could then be extracted from the grape berries into the wine during alcoholic fermentation, creating quality and sensory problems. Chapter 2 of this thesis gives an overview of the extensive literature that deals with methods of analysis of PAHs and malodorous phenols using both Gas Chromatography (GC), as well as High Performance Liquid Chromatography (HPLC). New methods for sample preparation, as well as analysis of PAHs using HPLC-Diode Array Detector (DAD), were developed and the results reported in Chapter 3. It was demonstrated that Sauvignon blanc experimental wines contained only chrysene at very low levels. The concentrations of chrysene found in the experimental wines are within the prescribed parameters as established by The Commission of the European Communities. Since no other PAH compounds were found in the samples analysed, it was concluded that the experimental wines were safe for human consumption and complied with EU regulations. The effect of vineyard posts on the sensorial characteristics of wine is discussed in Chapter 4. Creosoted poles were found to be responsible for an off-flavour described as ‘burnt rubber’ and ‘tarry’ in Merlot wines produced from grapes grown in close proximity to the posts.

Following some of the reported findings, new guidelines have been introduced in the Integrated Production of Wine certification, which advise against the use of creosoted poles for vineyard trellising. This preliminary but important guideline will bring the South African wine industry a step closer to the fulfilling the obligations for food safety as required by the legislation of our major export partners. Future investigations are recommended to completely understand and evaluate the cumulative effect of creosoted posts in a fully trellised vineyard.

# Opsomming

Oor die afgelope paar jaar is Suid-Afrikaanse wyne onder die soeklig geplaas as gevolg van verwysings in die internasionale wynmedia na ‘n duidelike ‘gebrande rubber’-karakter. Baie wynmakers en wyndeskundiges het aangedui dat hierdie besonderse karakter toegeskryf kan word aan wynbereidingsfoute wat verband hou met gisting wat wanbestuur is. ‘n Alternatiewe moontlike oorsprong van die smaak is geïdentifiseer in die koolteer wat as ‘n houtpreserveermiddel in wingerdopleistelsels gebruik word. Suid-Afrikaanse regulasies maak voorsiening vir die gebruik van kreosoteerde nutspale in landbougrond, hoewel hulle gebruik in Europa en die VSA hoogs gereguleerd en in sommige gevalle selfs verbied is. Die besmetting van kossoorte deur polisikliese aromatiese koolwaterstowwe (*polycyclic aromatic hydrocarbons (PAHs)*) is een van die vernaamste redes vir die verbanning van kreosoot in Europa en die VSA. Sommige van die verbindings in die kreosootmengsel het baie sterk geurkenmerke en daarom is dit die fokus van die huidige studie.

Die oorhoofse doelwit van hierdie studie was om te bepaal of wingerde wat op kreosoteerde pale opgelei is, die verskillende onwelriekende verbindings afkomstig van die houtbehandeling in die druiwekorrels kan akkumuleer of absorbeer. Hierdie verbindings sou dan tydens alkoholiese gisting uit die druiwekorrels in die wyn geëkstraheer kon word, wat aanleiding sou gee tot kwaliteits- en sensoriese probleme. Hoofstuk 2 van hierdie tesis verskaf ‘n oorsig van die breedvoerige literatuur wat handel oor metodes om *PAH*’s en onwelriekende fenole met behulp van beide gaschromatografie (GC) en hoëdrukvloeistofchromatografie (*HPLC*) te analiseer. Nuwe metodes is ontwikkel om monsters voor te berei en om *PAH*’s met behulp van ‘n *HPLC-diode array detector (DAD)* te analiseer. Die resultate word in Hoofstuk 3 gerapporteer. Daar is aangetoon dat die eksperimentele Sauvignon blanc-wyne chriseen teen baie lae vlakke bevat het. Die konsentrasies van chriseen wat in die eksperimentele wyne gevind is, is binne die voorgeskrewe parameters van die Kommissie van die Europese Gemeenskap. Aangesien daar nie ander *PAH*-verbindings in die geanaliseerde monsters gevind kon word nie, is daar tot die gevolgtrekking gekom dat die eksperimentele wyne veilig is vir menslike verbruik en aan die EG-regulasies voldoen. Die effek van wingerdpale op die sensoriese kenmerke van wyn word in Hoofstuk 4 bespreek. Kreosoteerde pale is gevind wat verantwoordelik is vir ‘n wangeur in Merlot-wyne afkomstig van druiwe wat naby die pale gegroei het en wat as ‘gebrande rubber’ en ‘teeragtig’ beskryf is.

Op grond van sommige van die gerapporteerde bevindings, is nuwe riglyne ingesluit in die sertifisering vir die Geïntegreerde Produksie van Wyn, wat aanbeveel dat kreosoteerde pale nie vir wingerdoplei gebruik word nie. Hierdie voorlopige, maar belangrike riglyn sal die Suid-Afrikaanse wynbedryf al beter in staat stel om te voldoen aan die voedselveiligheid regulasies wat vereis word deur die wetgewing van ons belangrikste uitvoervennote. Toekomstige ondersoeke moet

aangewend word om die kumulatiewe effek van kreosoteerde pale in volledig opgeleide wingerde ten volle te verstaan en te evalueer.

This thesis is dedicated to

*My family for their continuous support, encouragement and motivation*

# Biographical sketch

Valeria Panzeri was born on 3 March 1975 and matriculated at scientific high school A Messedaglia in Verona, Italy in 1994. She obtained her BSc-degree at Stellenbosch University in 2005, majoring in Viticulture and Oenology. After working for 5 years in the wine industry as winemaker, in 2011 Valeria enrolled for a MscAgric degree in Oenolgy at the University of Stellenbosch.

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# Preface

This thesis is presented as a compilation of 6 chapters.

**Chapter 1** **General Introduction and project aims**

**Chapter 2** **Literature review**

**Chapter 3** **Research results**
Chemical effects of vineyard posts on wine

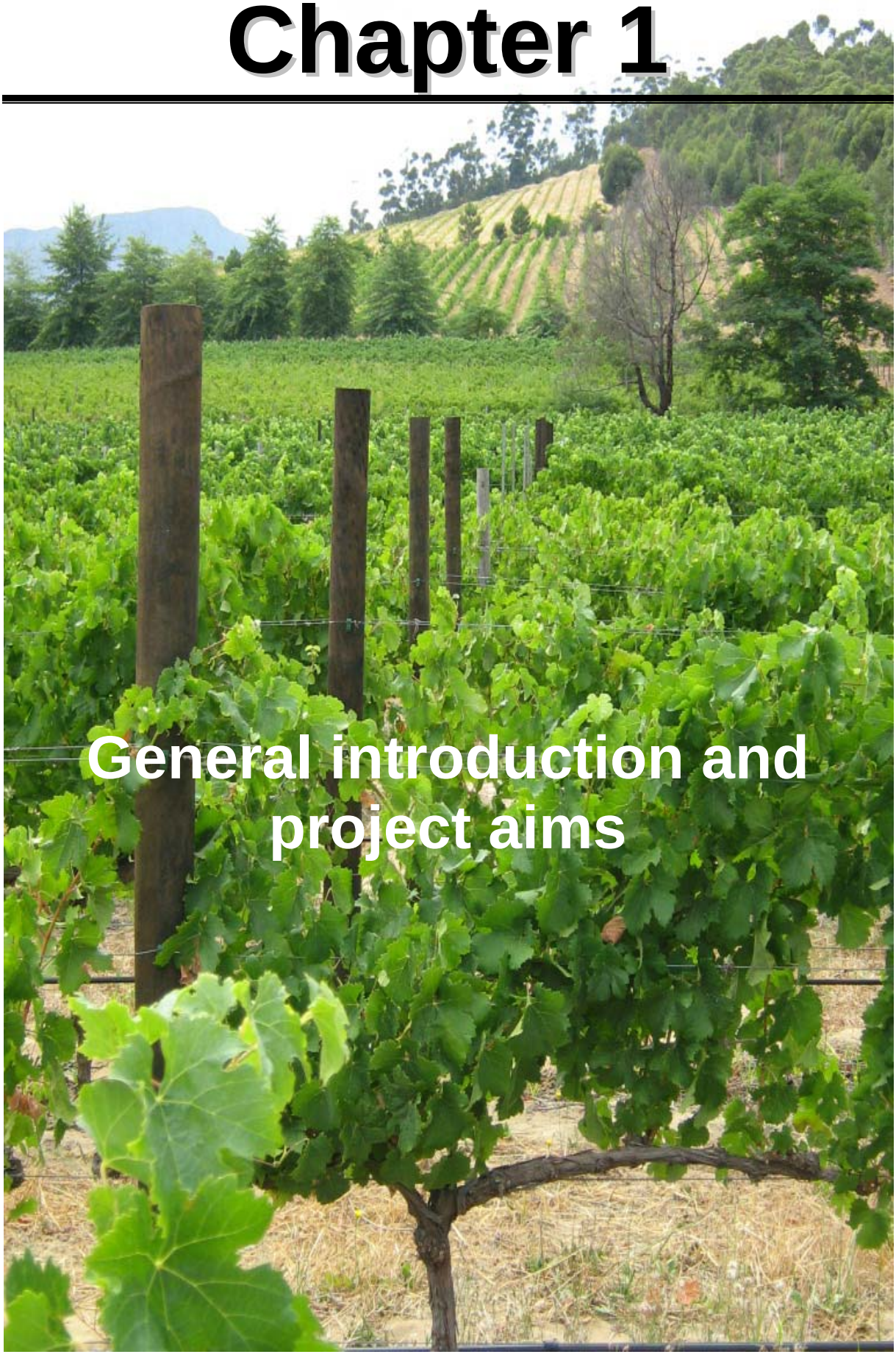
**Chapter 4** **Research results**
Sensory effects of vineyard posts on wine

**Chapter 5** **General discussion and conclusions**

**Chapter 6** **Appendices**

# Contents

# Chapter 1



## General introduction and project aims

# 1. GENERAL INTRODUCTION AND PROJECT AIMS

## 1.1 INTRODUCTION

In recent years South African wines have been under the spotlight due to an article published in *The Times* newspaper by Jane MacQuitty, a British journalist, who described them as having a distinctive 'burnt rubber' character, raising the doubt it could be a *terroir* derived taint (MacQuitty, J., 2008). Many winemakers and wine experts argued that the peculiar character could be ascribed to winemaking errors linked to mismanaged fermentation (Eedes, C., 2008), which may generate compounds such as ethyl mercaptan and diethyl disulfide described in literature as "burnt match and rubbery" (Swiegers and Pretorius, 2005). Although this theory has validity, a potential, different origin was identified in 2009.

During the harvest season of 2009, an Australian student working as winemaker assistant in South Africa, noted an intense and distinct 'tarry', pungent smell while driving near a creosote plant, which produced posts for trellis systems for the agricultural industry. He identified the odour as the possible culprit of the 'burnt rubber' character. From this initial anecdotal evidence, the Department of Oenology of the Stellenbosch University initiated a series of formal scientific projects aimed at investigating the potential link between the use of creosoted posts in vineyards and the 'burnt rubber' character of some of the South African wines.

Trellis systems, or training systems, are an integral part of the agricultural infrastructure, used for centuries to increase the yield and quality of various crops. A wide range of materials are used in viticulture for the production of supporting posts, the most common ones are wood, concrete and metal posts. Recent new alternatives include recycled plastic and combination of wood with reinforced metal covers. Pressure treated wooden posts are produced using different timber preserving substances: Copper Chromium Arsenate (CCA), Creosote, Copper azoles and Boron, just to mention few of them (Conradie, D. 2011).

Coal tar creosote is a wood preservative derived from the distillation of crude coal tar, which has been used for decades to protect utility poles against climatic and biological degradation. Coal tar creosote is a dark brown, thick liquid with a strong smoky or sharp aromatic odour. The composition of the creosote mixture is very complex and can differ depending on the origin of the coal and the method used for distillation. The number of chemical compounds in creosote can be as high as several hundred and can be divided into

six major classes: aromatic hydrocarbons, tar acids/phenolics, tar bases/nitrogen containing heterocycles, aromatic amines, sulphur containing heterocycles and oxygen-containing heterocycles (Melber, *et al.,* 2004). Some of the compounds in each class display strong odour characteristics (Choudhary, *et al.*, 2002) and it is for this specific reason that creosote became the focus of attention for the present study.

Creosote is an effective and economical material and therefore one of the favourite choices as wood preservative in South Africa (Eloff, 2000). Despite the fact that South African regulations allow for the use of creosoted utility poles in agricultural land (Standards, 2000), in Europe and USA their usage has been highly regulated and even banned for certain applications (The Commission of the European Communities, 2001; Dickey, 2003). Research shows how the phases necessary to confirm an environmental case could take decades to go from the detection of a problem to the actual establishment of regulations. Nevertheless, in the interim period, an 'As Low as Reasonably Achievable' (ALARA) principle should be adopted for the level of exposure for humans and the environment (Molhave, 2003). The delay in complying with international regulations and the presence of creosote in the agricultural sector in South Africa, could lead to terrible consequences in case this would be brought to the attention of the media. Contamination of the foodstuff, as well as health risks for the workers who handle the creosote products, has been the major motives for the banning of creosote in Europe and USA. International awareness of the inadequacy of our policies could lead South African agricultural sector and the wine industry to collapse.

## 1.2 PROJECT AIMS

This project forms part of a group of studies conducted by the Department of Oenology and Viticulture, focused on the determination of Polycyclic Aromatic Hydrocarbons (PAHs) deriving from environmental pollution and human activities, their degree of assimilation by the plant and possible transfer to the final wine product.

The main aim of this study is to determine if vines trellised with creosoted posts, could accumulate or absorb the various malodorous compounds on the waxy layer of the fruit, transferring the contaminants to the wine. The accumulation of phenols, cresols, xylenols and PAHs on the grapes could lead to the extraction of the unwanted compounds into the wine during alcoholic fermentation, creating quality and sensory problems. To establish a direct link between the creosote exposure and the characteristic 'burnt rubber' taint, various chemical and sensorial analyses were combined with statistical techniques. The outcome of this project will identify the possible source of the 'burnt rubber' taint in South African wines

and help the producer to avoid the unwanted character in the final products. Furthermore, it might provide the South African agricultural industry with strong evidence against the use of creosoted utility posts, already banned in other countries around the world.

Other aims of the project were as follows:

a) Assess differences in the sensorial and chemical characteristics of wines made from grapes grown adjacent to metal, new creosote and 10 year old creosoted poles. The establishing of a trial including recycled plastic posts has been added in the 2011/2012 growing season but at this stage data are not sufficient for a comprehensive evaluation.
b) Develop a method to analyze and quantify creosote related compounds in an alcoholic matrix, using HPLC-DAD.
c) Identify the presence of PAHs and other creosote related compounds in wines, establishing if there is a link between the use of creosoted posts and the transfer of those contaminants to the wines;
d) Verify if different winemaking practices (*i.e.* extent of skin contact) have an effect on levels of compounds detected;
e) Assess if there is a reduction in any compounds detected over a three year period.
f) Evaluate the experimental wines sensorially to assess the quality and ascertain if there is any association between creosote use and specific taints in wine.

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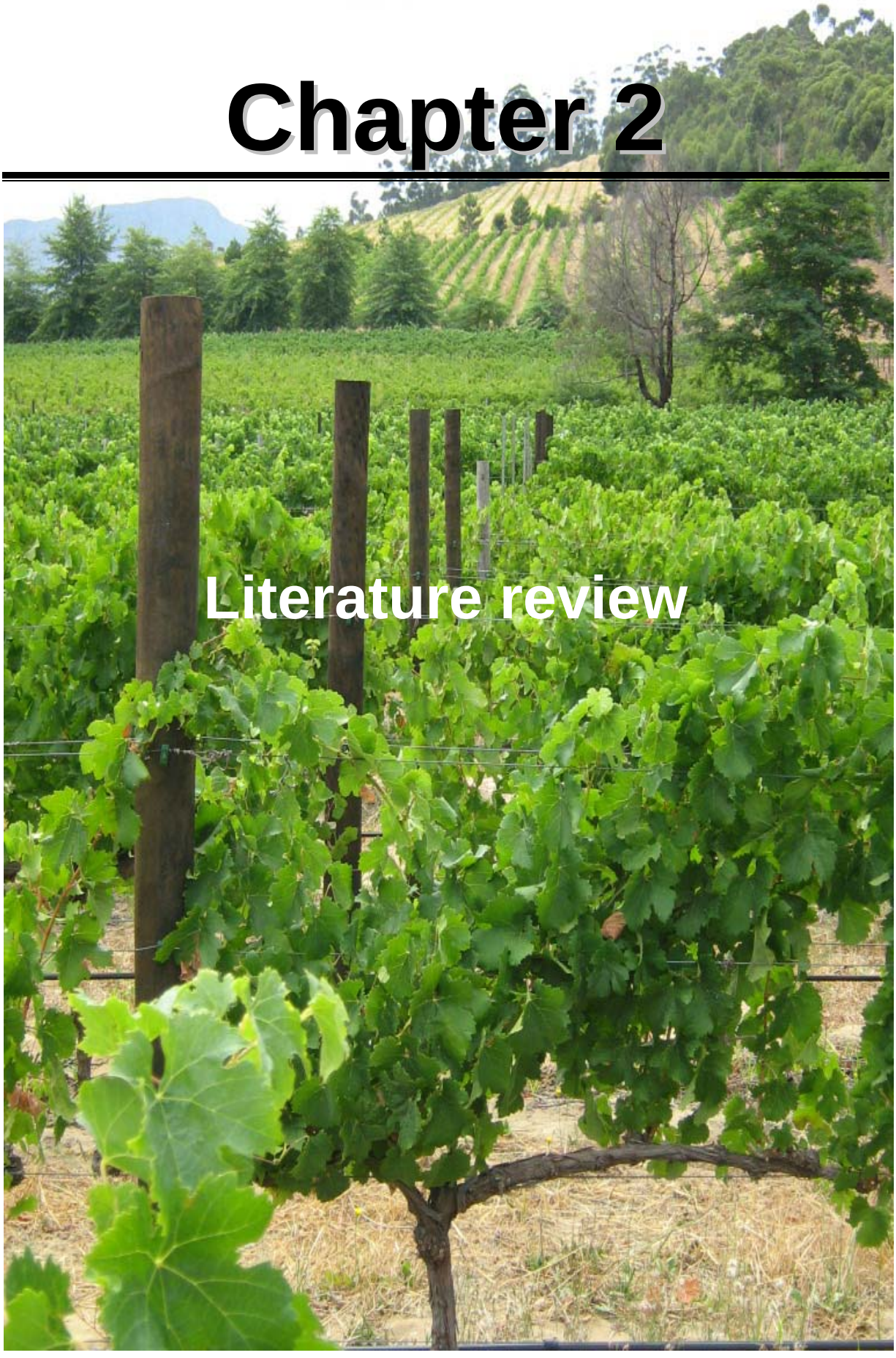
# Chapter 2



## Literature review

# 2. LITERATURE REVIEW

## 2.1 INTRODUCTION

Trellis systems, or training systems, are an integral part of the agricultural infrastructure, used for centuries to increase the yield and quality of various crops. Early proof of their use is found already in Romans times, where authors such as Columella write in his book *De Re Rustica* about the advantages of training the grapevine. Training systems used today in agriculture include posts, wires and other structures which help sustaining the plant during his growing season.

A wide range of materials are used in viticulture for the production of supporting posts. The most common ones are wood, concrete and metal posts. Recent new alternatives include recycled plastic and combination of wood with reinforced metal covers. Pressure treated wooden posts are produced using different timber preserving substances, including copper chromium arsenate (CCA), creosote, copper azoles and boron, (Conradie, D., 2011). Choosing the most suitable material for utility poles has been traditionally a matter of economical factors as well as durability and efficiency. In the United States guidelines have been put in place for the selection of wood preservatives accompanied by recommendations regarding the usage of these products in relation to human health (Dickey, 2003).

Creosote is still, nowadays, a very effective and economical material and therefore one of the favourite choices as wood preservative in South Africa (Eloff, A., 2000). Despite the fact that South African regulations allow for the use of creosoted utility poles in agricultural land as long as they comply to the South African Bureau of Standards (SABS 457,1994), in Europe and USA their usage has been highly regulated and even banned for certain applications (Commission Directive 2001/90/EC; Dickey, 2003).

The concerns related to creosoted posts are two-fold: firstly extensive literatures identifies in creosote a source of human health hazards ranging from carcinogenetic (Environmental Protection Agency, 2011) (Registry, 1995) (Scientific Committee on Food, 2002) (Agency for Toxic Substances and Disease Registry, 2002) to toxicity (Environmental Protection Agency, 2011) to environmental pollutant (Busetti, 2006) (Minoia, 1997) (NPI Australia, 2004); secondly, anecdotal evidences relate the strong, pungent smell of this tarry product to a ‘burnt rubber’ character of certain South African wines.

## 2.2 CREOSOTE

### 2.2.1 Physical and chemical characteristics

Coal tar creosote is a wood preservative derived from the distillation of crude coal tar, which has been used for decades to protect utility poles against climatic and biological degradation. Coal tar creosote is a dark brown, thick liquid with a strong smoky or sharp aromatic odour. The composition of the creosote mixture is very complex and can differ depending on the origin of the coal and the method used for distillation (Melber, 2004). The number of chemical compounds in creosote can be as high as several hundred and can be divided in six major classes: aromatic hydrocarbons (PAHs), tar acids/phenolics, tar bases/nitrogen containing heterocycles, aromatic amines, sulphur containing heterocycles and oxygen-containing heterocycles. Some of the compounds in each class display strong odour characteristics (Choudhary *et al*., 2002).

Table 2.1 summarizes the odour characteristics of the compounds of interest, separating them according to the group under which they are classified.

**Table 2.1** Creosote compounds with distinct odour characteristics.

| CLASS | COMPOUND | ODOUR | References |
|---|---|---|---|
| **PAHs** | Acenaphthene | tar | |
| | Benzothiophene | solvent, rubbery, earthy | The Good Scent Company n.d. |
| | Naphthalene | moth balls, tarry | The Good Scent Company n.d. |
| **Volatile Organic Compounds** | Benzene | sweet, aromatic | Environmental Protection Agency, 2012 |
| | Toluene | paint thinner | Environmental Protection Agency 2012 |
| | Xylenes | sweet | Hazard Evaluation System and Information Service, 1989 |
| **Tar acids/phenolics** | Phenol | smoky, medicinal | Chattonet |
| | 4-Ethylphenol | phenolic, pungent | Chatonnet *et al*., 1992 |
| | o-Cresol | medicinal, smoky | Parker *et al*., 2010 |
| | m-, p-Cresol | medicinal | Parker *et al*., 2010 |
| | 3,4-dimethylphenol | sick sweet, medicinal | Burdock, 2010 |
| | 2,3-dimethylphenol | chemical | Burdock, 2010 |
| | 2,5-dimethylphenol | creosote, medicinal | Burdock, 2010 |

Table 2.1 (cont.)

| CLASS | COMPOUND | ODOUR | References |
|---|---|---|---|
| **Tar bases** | Indole | faecal | Budavari 1996 |
| **N containing heterocycles** | Quinoline | tar | The Good Scent Company n.d. |
| | Benzoquinoline | pungent/irritating | |
| | Methylcarbazole | naphtalene | |
| | Acridine | irritating | |
| **Aromatic amines** | Aniline | rotten fish | Castellani, 2002 |
| **S containing heterocycles** | Benzothiophene | moth balls | The Good Scent Company n.d. |

The main groups considered in the present study are:

- volatile phenols: 4-ethylphenol, phenol, cresols and xylenols.
- Polycyclic Aromatic Hydrocarbons (PAHs)

### 2.2.2 Volatile phenols: chemical and physical characteristics

Volatile phenols are a class of compounds which includes many sub-groups such as ethylphenols, vinylphenols, cresols and xylenols. Many of those compounds are an integral part of the aroma profile of wine, but can represent a threat to good quality if present at high concentrations. Chatonnet *et al*. (1992) showed that volatile phenols are produced during malo-lactic fermentation by yeast of the genus *Brettonomyces:* cinnamate decarboxylase enzymes convert cinnamic acids into volatile phenols by non-oxidative decarboxylation. In particular, *p*-coumaric acid, present in grapes, is converted into 4-vinylphenol during alcoholic fermentation by *Saccaromyces cerevisiae.* Later a further step can occur, if *Brettonomyces* species are present in the wine, and 4-vinylphenol gets converted into 4-ethylphenol by vinyl-phenol reductase. Table 2.2 below shows odour thresholds (OT) of some of the volatile phenols according to literature.

**Table 2.2** Some volatile phenols compounds, their concentration, threshold values and odours.

| Compound | Odour threshold (µg/L) | Odour | Reference |
|---|---|---|---|
| **4-ethylphenol** | 605[1] | wet horse, animal | Chatonnet *et al*., 1992 |
| **phenol** | 7100[2] | artificial sweetness | HPA* Parker *et al*., 2010 |
| **p-cresol** | 10[2] 3.9[3] | chemical, tar-like, mothballs | HPA* Parker *et al*., 2010 |
| **o-cresol** | 31[2] | smoky, tar-like | Parker *et al.,* 2010 |
| **m-cresol** | 68[2] 15[3] | medicinal | Parker *et al*., 2010 |
| **3,4 Xylenol** | 1200[2] | sick sweet | Burdock 2010 |

(*) Health Protection Agency – Odour complaints checklist (2011)

([1]) DT in red wine

([2]) Odour DT in aqueous solution at 10% ethanol

([3]) Odour DT in water

Cresols and xylenols are found in small amounts in various products: essential oils, tea, roasted coffee and wine (Merisol, 2009). Fernandez de Simon *et al.* investigated the presence of those compounds in wine and linked their origin to oak wood pyrolysis (Fernandez de Simon, Cadahiia, del Alamob, & Nevaresb, 2010). 3,4-xylenol (or 3,4-dimethylphenol) was detected in wine treated with toasted American oak (Kaushal, 2007), but in a preceding study by Etievant (Etievant, 1981) cresols and xylenols were found in unwooded wines, demonstrating that wood treatment was not solely responsible for their occurrence in the finished product. Recently, grapes and wine affected by smoke have shown to contain volatile phenols, including o-cresol and phenol (Hayasaka et al., 2010). Cresols, xylenols and phenols are also known in the chemical industry as cresylic acid, a mixture used in wood preservative products, like creosote, used for utility poles (Merisol, 2009).

#### 2.2.2.1 Volatile phenols: method of analysis

Volatile phenols compounds have been a challenge for the wine industry for many years. The aroma deriving from *Brettanomyces/Dekkera* spoilage is associated with 'medicinal', 'animal', 'smoky' and 'barnyard' smell, surely unwanted characters in quality wines. The importance of methods of analysis which can be conducted fast and with accuracy is therefore relevant. Various methods can be used, ranging from liquid chromatography (LC) to gas chromatography (GC) and coupled with mass spectrometry (MS) for identification of unknown compounds. One of the most time consuming aspects of the analysis is the sample preparation, which often involves multiple steps involving use of expensive and hazardous solvents. Monje *et al*., (2001) demonstrated the efficiency of Head Space Solid Phase Micro Extraction (HS-SPME) coupled with GC in determining ethylphenols in wine. The advantages of HS-SPME compared to Liquid Liquid Extraction (LLE) are the use of a small volume of samples, rapidity and high sensitivity; one disadvantage is that the method is less selective than LLE and therefore the chromatograms present more peaks. This problem can be overcome by the MS component of the system which allows for peak identification.

### 2.2.3 Polycyclic Aromatic Hydrocarbons

#### 2.2.3.1 Chemical and physical properties

PAHs are found in nature as a group, and not as individual compounds, resulting from pyrolysis of organic material. As pure single chemicals they can be colourless, white or pale-yellow with a faint odour. According to the International Union of Pure and Applied Chemistry (IUPAC), PAHs contain three or more benzene rings, but naphthalene is often also include in the group and it is characterized by a strong, pungent mothball odour (National Pesticides Information Centre, Naphthalene technical fact sheet).

PAHs possess characteristic and unique ultraviolet (UV) spectra which are available in various databases and enable identification of individual chemicals (*e.g.* http://webbook.nist.gov) when analyzed with UV diode array detectors (DAD) or photo diode array (PDA).

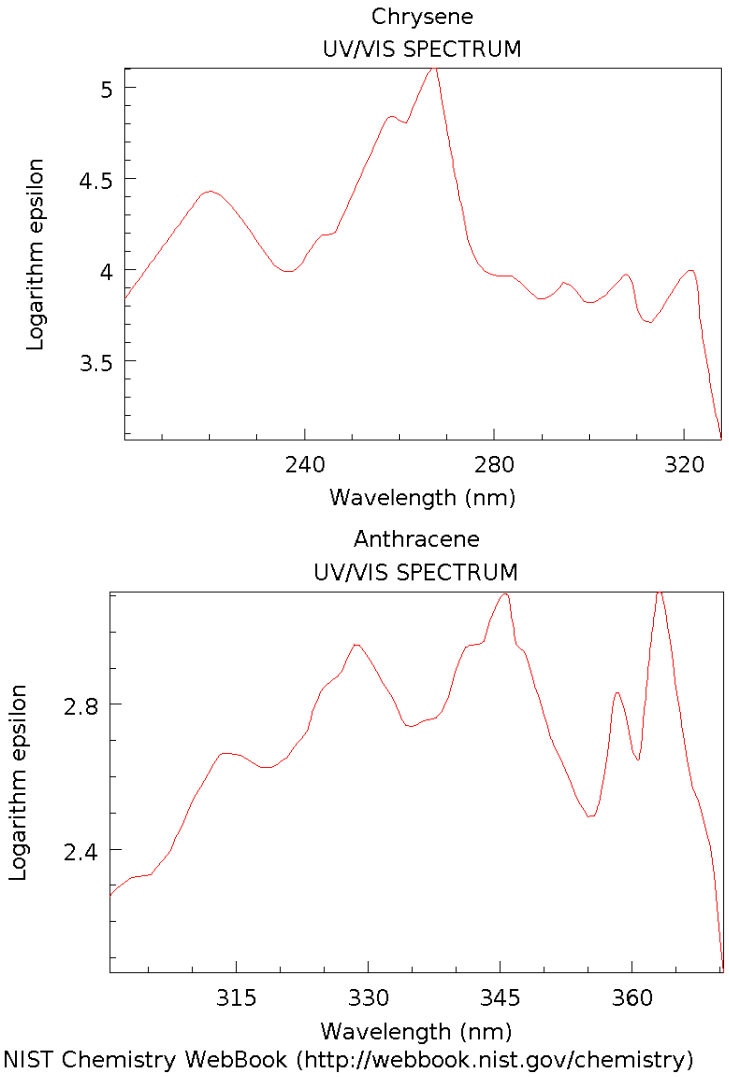



**Figure 2.1** UV/Vis spectra of Chrysene and Anthracene (National Institute of Standards and Technology, 2010).

Another important property of some PAHs is fluorescence**.** When expose to an external light source (lamp or laser) the photons are absorbed by the fluorophore (PAH molecule) creating an excited electronic state. After being on the excited state for a period of time, where some of the energy is dissipated, a photon is emitted and the flourophore returns to his ground state. The difference between excited and emitted wavelengths (or energy) can be measured with fluorescence detectors (FLD). This method of analysis is the most commonly applied in the study of PAHs since, due to its high sensitivity, it allows for quantitation at part per billion levels.

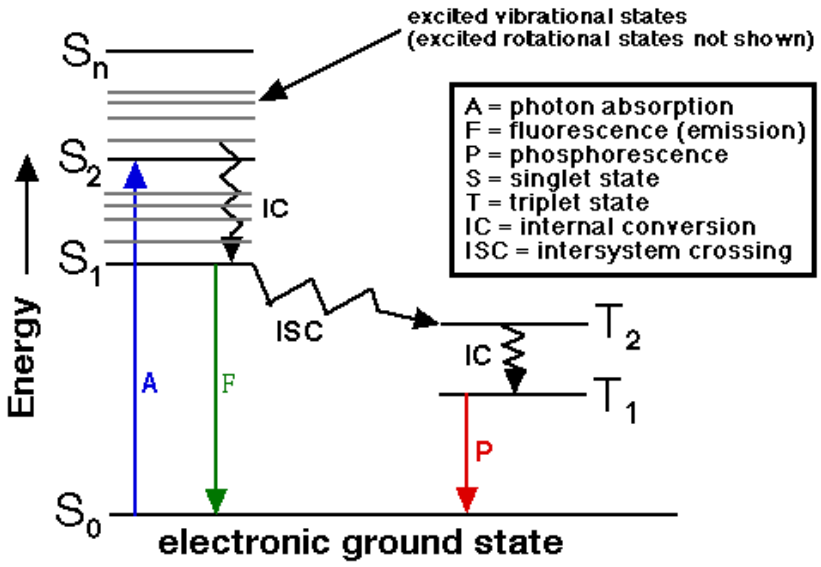



**Figure 2.2** Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence (www.shsu.edu/~chm_tgc/chemilumdir/JABLON.GIF).

The solubility of PAHs in aqueous matrix decreases with the increase in the number of benzene rings: the larger the molecule is the more non-polar the molecule is. Acenaphthene and acenaphthylene, (3 benzene rings structure) water solubility is 1.93 mg/L and 3.93 mg/L respectively, while it is only $2.3 \times 10^{-3}$ mg/L for B[a]P (5 benzene rings structure) (ATSDR, Toxicological profile for PAHs, 1995). This gives rise to important considerations from a chemical and physical point of view: the lipophilic nature of PAHs enables them to be easily adsorbed onto the cuticle layer of vegetation (Kipopoulou, 1999); the non-polarity determines the choice of stationary and mobile phases in HPLC analysis.

Since volatility in PAHs is mostly very low, the presence of PAHs in the atmosphere is linked to their interaction with the various aerosols present in the air. Temperature also plays an important role which has been reported to account for 21–67% of the variability in gas-phase concentrations (Sitaras, 2004).

Effective degradation mechanisms for PAHs are photo-oxidation (Neff, 1979), chemical oxidation (Ferrarese, 2008) and biodegradation by microorganisms (Johnsen *et al.*, 2005), but the relevance of each process depends on specific conditions, like oxygen availability in the medium and temperature. The rate of degradation and the importance of each mechanism are not related to the molecular size of the compounds.

#### 2.2.3.2 PAHs in the environment and in foodstuff

PAHs are not only found in creosote and in oil-derived products. Various sources have been determined, which include smoke produced by bush fires (Radojevic, 2003; Kennison, 2009) or deriving from human activities such as: grilling or smoking of food product (Bocca, 2003; JECFA, 2005), burning of fuels and tobacco or pollution caused by oil spillages or leaching of coal tar products (Bedient, 2004). Their impact is therefore widely spread and PAH compounds can be found in contaminated water, soils and air, as well as food products such as fish, smoked products, fruits and vegetables grown in vicinity of contaminated sites (Bocca, 2003; Corradetti, 2002; Moret, 2007).

Several studies focused on plant uptake of those pollutants from either the above ground parts or the root system (Bohme, 1999). A study on carrots, potatoes and lettuce indicated that low molecular weight PAHs (less than 6 rings) seemed to be taken up by the plant through the atmosphere into the leaves due to their more volatile nature, while high molecular weight ones, such as benzo[a]pyrene, used as preferred pathway the root system (Fismes, 2002). PAHs are lipophilic molecules and therefore likely to be absorbed into the waxy cuticle layers of leaves, but they can also be taken up in gaseous phase through the stomata (Kipopoulou, 1999).

Attention is also been given in the literature to other 5 classes of compounds which are present in the creosote mixture, namely tar acids/phenolics, tar bases/nitrogen containing heterocycles, aromatic amines, sulphur containing heterocycles and oxygen-containing heterocycles. They do not constitute such a high percentage of the creosote mixture (approximately 5-10%), but they are more soluble in water and therefore they are found in high percentage in contaminated water, soil and sediments (Choudhary *et al*., 2002).

#### 2.2.3.3 International and national legislation

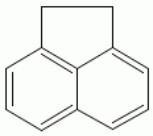
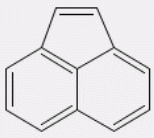
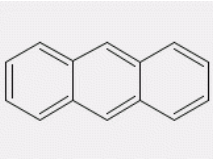
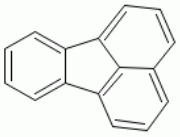
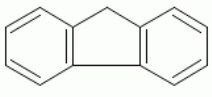
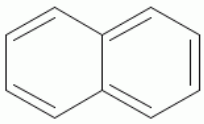
In the past decades PAHs have been the subject of intense investigation due to the fact that some of them have been proved to be toxic (USDHTP, 2002). The International Agency of Cancer (IARC) identified 12 PAHs as being carcinogenic to humans based on studies conducted on animals between 1973 and 1987 (http:/www.iarc.fr./ENG/Database/index.php). In the United States of America, the Environmental Protection Agency (US EPA), Office of Environmental Health Hazards (OEHHA) publishes every year a list of chemicals known to cause cancer which has its roots in the 'Safe Drinking Water and Toxic Enforcement Act' of 1986 (OEHHA, 1986, Proposition 65). Wenzl *et al.,* in a review published in 2006, highlighted the need of further investigation and method development of the 16 EU priority PAHs and the necessary creation of standardized procedures for routine analysis in various

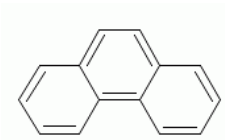
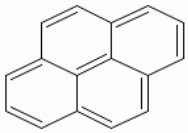
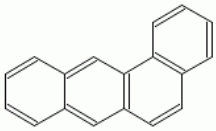
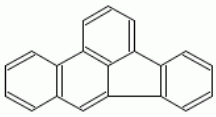
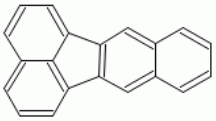
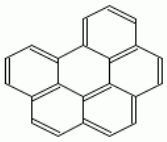
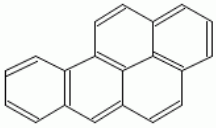
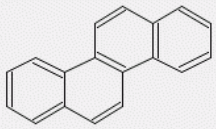
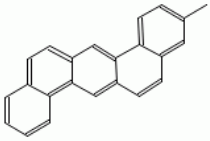
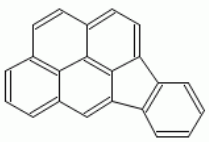
foodstuff. Table 2.3 below includes the 16 PAHs and their abbreviations as reference for the remaining of this document.

**Table 2.3** 16 PAHs (EPA) and their abbreviations.

| PAH | Abbreviation | PAH | Abbreviation |
|---|---|---|---|
| Acenaphthene | Ace | Chrysene | Chr |
| Acenaphthylene | Acy | Dibenz[a,h]anthracene | DBahA |
| Anthracene | Ant | Fluoranthene | Fla |
| Benzo[a]anthracene | BaA | Fluorene | Fluo |
| Benzo[a]pyrene | BaP | Indeno[1,2,3-cd]pyrene | IP |
| Benzo[b]fluoranthene | BbF | Naphthalene | Naph |
| Benzo[g,h,i]perylene | BghiP | Phenanthrene | Phe |
| Benzo[k]fluoranthene | BkF | Pyrene | Pyr |

**Table 2.4** Molecular structures of PAHs of concern and their relevance according to different regulations (adapted from Wenzl *et al*., 2006).

| PAHs | Structure | AMU[a] | US-EPA[b] | SCF[c] | JECFA[d] |
|---|---|---|---|---|---|
| Acenaphthene |  | 154 | X | | |
| Acenaphthylene |  | 152 | X | | |
| Anthracene |  | 178 | X | | |
| Fluoranthene |  | 202 | X | | |
| Fluorene |  | 166 | X | | |
| Naphthalene |  | 128 | X | | |

| | | | | | |
|---|---|---|---|---|---|
| Phenanthrene |  | 178 | X | | |
| Pyrene |  | 202 | X | | |
| Benzo[a]anthracene |  | 228 | X | X | X |
| Benzo[b]fluoranthene |  | 252 | X | X | X |
| Benzo[k]fluoranthene |  | 252 | X | X | X |
| Benzo[g,h,i]perylene |  | 276 | X | X | |
| Benzo[a]pyrene |  | 252 | X | X | X |
| Chrysene |  | 228 | X | X | X |
| Dibenz[a,h]anthracene |  | 278 | X | X | X |
| Indeno[1,2,3-cd]pyrene |  | 276 | X | X | X |

[a] Atomic Mass Units, [b] United State Environmental Protection Agency, [c] Scientific Committee on Food,

[d] Joint FAO/WHO Expert Committee on Food Additives.

Methods of sampling and analysis in food have also been regulated by the EU (EC No 333/2007. 2005/10/EC) and the International Organization for Standardization is currently working on the preparation of enlarged sets of PAHs in different matrices. Two of the ISO methods use High Performance Liquid Chromatography (HPLC) with fluorescence detectors (ISO 15302, ISO/AWI 22959) while a third method (ISO/AWI 24054) uses Gas Chromatography Mass Spectroscopy (Wenzl *et al*., 2006), but all three of them focus only on a portion of the 16 PAHs of interest outlined by US EPA, the Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Table 2.4). The Commission of the European Communities in directive EC 1881 of 2006 chose Benz[a]pyrene (B[a]P) as a marker to monitor the level of PAHs contamination in foodstuff (Table 2.5). Alcoholic beverages are not included in the particular legislation, but maximum limits of B[a]P allowed are 2.0 µg/kg of wet weight (or parts per billion).

**Table 2.5** Maximum acceptable levels of Benzo[a]pyrene in foodstuffs (adapted from EC 1881/2006, Annex Section 6 – Benzo[a]pyrene).

| Foodstuffs | Maximum levels (µg/kg wet weight) |
|---|---|
| Oils and fats (excluding cocoa butter) intended for direct human consumption or use as an ingredient in foods | 2.0 |
| Smoked meats and smoked meat products | 5.0 |
| Muscle meat of smoked fish and smoked fishery products, excluding bivalve molluscs. The maximum level applies to crustaceans, excluding the brown meat of crab and excluding head and thorax meat of lobsters and similar large crustacean (*Nephropidae* and *Palinuridae*) | 5.0 |
| Muscle meat of fish, other than smoked fish | 2.0 |
| Crustaceans, cephalopods, other than smoked. The maximum level applies to crustaceans, excluding the brown meat of crab and excluding head and thorax meat of lobsters and similar large crustacean (*Nephropidae* and *Palinuridae*) | 5.0 |
| Bivalve molluscs | 10.0 |
| Processed cereal-based foods and baby foods for infants and young children | 1.0 |
| Infant formulae and follow-on formulae, including infant milk and follow-on milk | 1.0 |
| Dietary foods for special medical purposes intended specifically for infants | 1.0 |

South African legislation allows for the use of coal tar creosote as wood preservative and no restrictions are in place for its use. Creosote is sold in hardware stores and, apart from warning signs on the labels and instruction for its use, anybody can purchase it and apply it either for outdoor or indoor wood treatment. .

The South African Bureau of Standards (SABS) and the South African Wood Preservers Association (SAWPA) are bodies responsible for setting standards for the preservation of wood. Those associations work hand in hand with the Forestry Department and their interest lies in protection of the South African timber. Standards are set according to the final usage of the lumber and therefore regulate the type of preservative most suited and the necessary parameters to ensure the wood durability in respect to environmental conditions and biological degradation (Eloff, 2000).

**Figure 2.3** Certified SABS 457 creosoted posts (Photo: http://www.timberdirect.net/creosote-pine-poles.htm)

The SABS 457 is of relevance for the wine industry since it regulates the trellis system posts: strength, cosmetic and treatment requirements. The latter ensure that each pole receives the right amount of wood preservative and penetrates into the grain of the timber sufficiently to ensure protection from environmental and biological degradation (South African Bureau of Standards).

There is at present no legislation related to health or environmental pollution issues. In July 2011 the Integrated Production of Wine (IPW) certification system introduced in their guidelines a paragraph suggesting the usage of creosote alternatives as wood preservative for use in the vineyards. This addition to the guiding principles for a sustainable production in the wine industry was strongly influenced by the proceeding of the present study and the realization of the outdated legislative situation of South Africa compare to the EU and USA countries.

Wine sales to the export market reached 49% of total sales in 2012 (Retief, 2012) and great importers of our beverages are many EU countries and US in which PAHs are highly monitored in foodstuff. The relevance of the issue is such that it cannot be ignored any longer. Creosoted posts in the vineyards have been banned for so many years in the EU and USA that a systematic analysis of alcoholic beverages is not been consider necessary. The need for the SA industry to monitor the local production of wine to ensure a safe product is of the outmost importance: the entire export market could be jeopardized.

Furthermore, the importance of sustainable viticulture which preserves biodiversity in South Africa is at the top of the agenda of organizations like Biodiversity Wine Initiative (BWI) to which many wineries are adhering. Belonging to BWI and actively participating in a process of preservation of the unique Cape Floral Kingdom is a very important cause in which the producers believe. Furthermore, BWI certification is a powerful marketing tool which promotes SA as an advocate of biodiversity, favoring the image of the local wine industry overseas (Hall, 2008).

#### 2.2.3.4 Methods of analysis for PAHs

As reported by Douben in his book 'PAHs: an ecotoxicological perspective' (2003), this class of compounds have been subject of environmental research since 1940 when Kern discovered chrysene in soil samples from a German site and linking its source to pyrogenic material including coal. Since then, countless papers have been published on the presence of PAHs in the environment due to their established carcinogenic and mutagenic characteristics. As a group of compounds they are found everywhere in the natural surroundings, from water sources, to soil, to the atmosphere, but mainly as a result of human activities.

A system of PAH monitoring in foodstuff started in 2001 in the European Union, when analytical controls on olive oil pomace, destined for human consumption, revealed elevated amount of these compounds (Purcaro *et al.*, 2008). Since then, the need for a method of analysis which could be both effective and rapid has been of great importance. The relevant aspect to keep in mind is that any method which is developed has to comply with performance criteria as outlined by European Commission Directive 2005/10/EC: Benz[a]pyrene limit of detection (LOD) has to be superior or equal to 0.3 microgram per kilogram (µg/kg) and the limit of quantitation (LOQ) above the 0.9 µg/kg value. Another important parameter is the recovery percentage which can be included between 50 and 120% (Table 2.6).

**Table 2.5** Performance criteria for methods of analysis for BaP (adapted from 2005/10/EC).

| Parameter | Value/comment |
|---|---|
| Applicability | Food specified in Regulation (EC) No…/2005 |
| Detection limit | No more than 0.3 µg/kg |
| Limit of quantification | No more than 0.9 µg/kg |
| Precision | $HORRAT_r$ or $HORRAT_R$ values of less than 1.5 in the validation collaborative trial |
| Recovery | 50% - 120% |
| Specificity | Free from matrix or special interferences, verification of positive detection |

### 2.2.3.5 Sample storage

European directives are very specific on criteria for handling of organic material destined for analysis. It is clearly stated that "Wherever possible, apparatus coming into contact with the sample should be made of inert materials *e.g*. aluminium, glass or polished stainless steel. Plastics such as polypropylene, PTFE *etc*. should be avoided because the analyte can absorb onto these materials" (The Commission of the European Communities, 2005. Commission Directive 2005/10/EC). PAHs can also be subject to biological degradation and therefore collected samples need to be analyzed soon after collection or correctly stored at low temperatures. Benzo[a]pyrene levels in tap water showed a decrease of up to 60% when exposed to daylight and room temperature (García-Falcón *et al*., 2004). A study by Rila (2007) focused on the recovery of various PAHs from contaminated soil samples after storage at room temperature, -4ºC and -18ºC; a comparison was also made between glass and polyethylene containers. The results clearly showed that it is better to store samples in glass bottles at -18ºC. Recommendation for storage time for soil samples was determined to be of maximum 3 months. It is also important to note that amber, or dark glass, is most suitable for storage due to the sensitivity of some PAHs to photo-degradation.

### 2.2.3.6 Sample preparation

Several techniques exist for sample preparation. Many of them have been standardized for extraction of PAHs in different matrices. They include Solid Phase Extraction (SPE), Solid Phase Micro Extraction (SPME) and Liquid-Liquid Extraction (LLE). The most appropriate sample clean up to be used is dependent on the chromatographic technique, the matrix to be analysed and on the analyte of interest.

García-Falcón *et al.* (2004) conducted a study to determine the recovery of heavy PAHs in spiked water samples, utilizing SPE and comparing it with SPME technique. As shown in Table 2.7, the results showed that absolute recovery was higher for SPE. The precision,

expressed as relative standard deviation (RSD) was sufficient for both methods but SPME didn't reach levels satisfactory enough for the European regulation for drinking water. Furthermore, the sensitivity of SPE was higher than SPME since the recovery percentages were about 20 times higher for the former. Detection and quantitation limits were insufficient for SPME according to EU legislation.

**Table 2.7** Comparison between the performances of SPE vs. SPME techniques in respect of PAHs determination in spiked tap water (adapted from García-Falcón *et al*., 2004).

**Recovery, Repeatability, Linear Dynamic Ranges, Determination Coefficients ($r^2$), and Limits of detection (LOD) and Quantitation (LOQ) of the techniques for determining PAHs in tap waters**

(A) Solid-phase extraction

| | Absolute recovery [a] | | | Instrument Linearity [b] | | | |
|---|---|---|---|---|---|---|---|
| PAHs | Spiking level (ng/L) | % | ±% RSD | Range (µg/L) | $r^2$ | LOD[a] (ng/L) | LOQ[a] (ng/L) |
| Fl | 8.7 | 102 | 4 | 0.2-8.0 | 0.9994 | 0.2 | 0.5 |
| B[a]A | 2.2 | 97 | 4 | 0.15-3.0 | 0.9995 | 0.1 | 0.3 |
| B[e]P | 30.0 | 98 | 4 | 0.7-35 | 0.9995 | 0.7 | 1.5 |
| B[b]F | 8.6 | 101 | 2 | 0.5-6.0 | 0.9992 | 0.4 | 1.0 |
| B[k]F | 2.0 | 99 | 1 | 0.7-1.0 | 0.9997 | 0.05 | 0.15 |
| B[a]P | 4.0 | 96 | 2 | 0.15-4.0 | 0.9994 | 0.1 | 0.3 |
| D[a,h]A | 8.0 | 96 | 1 | 0.3-5.0 | 0.9997 | 0.2 | 0.6 |
| B[g,h,i]P | 23.0 | 99 | 2 | 0.5-20 | 0.9994 | 0.6 | 1.0 |
| I[1,2,3-cd]P | 26.0 | 95 | 3 | 0.7-35 | 0.9997 | 0.7 | 1.5 |

(B) Solid-phase Microextraction

| | Absolute recovery [a] | | | Instrument Linearity [c] | | | |
|---|---|---|---|---|---|---|---|
| PAHs | Spiking level (ng/L) | % | ±% RSD | Range (µg/L) | $r^2$ | LOD[a] (ng/L) | LOQ[a] (ng/L) |
| Fl | 70 | 5 | 4 | 20-175 | 0.9968 | 6 | 20 |
| B[a]A | 18 | 5 | 3 | 10-440 | 0.9905 | 3 | 10 |
| B[e]P | 140 | 8 | 6 | 80-600 | 0.9990 | 27 | 80 |
| B[b]F | 70 | 7 | 6 | 40-175 | 0.9925 | 13 | 40 |
| B[k]F | 18 | 7 | 6 | 10-440 | 0.9957 | 3 | 10 |
| B[a]P | 35 | 7 | 5 | 20-90 | 0.9999 | 6 | 20 |
| D[a,h]A | 70 | 8 | 8 | 40-175 | 0.9996 | 13 | 40 |
| B[g,h,i]P | 140 | 7 | 6 | 80-350 | 0.9989 | 27 | 80 |
| I[1,2,3-cd]P | 185 | 7 | 7 | 100-600 | 0.9985 | 37 | 100 |

[a] n = 7 determinations, [b] n = 10 in duplicate determinations, [c] n = 5 in duplicate determinations.

The same authors conducted analysis on alcoholic beverages with similar results for SPE (García-Falcón *et al.,* 2005): conditioning of the octadecyl mini-column (C18) with methanol and water was performed; the C18 was then loaded with wine sample, at a rate of 5 mL/minute, and subsequently flushed with acetonitrile and water (20:80). The column was dried using a stream of nitrogen and then connected to a silica column where the PAHs collected by hexane washing. The aliquot was finally evaporated under nitrogen and re-dissolved in acetonitrile for HPLC analysis. Also in this case the proposed method produced good results allowing for detection at ng/L levels. Variations to the SPE technique include the use of different solvents like *n*-hexane and dichloromethane for elution of PAHs in vegetable oil matrix (Purcaro *et al.,* 2008; Moret and Conte, 2002).

The QuEChERS methods from Agilent Technologies have been used for sample preparation (application notes from Agilent Technologies). The QuECHERS methods are modified versions of the SPE technique and can be applied to organic matrices such as soil, water, plant material and other biological matrices. The technique includes a two step process: QuEChERS 50 mL Teflon centrifuge tubes are used for the first extraction step in which the homogenized sample is placed with acetonitrile addition; a salt packet, composed of a pre-weight $MgSO_4$ and $C_2H_3NaO_2$ is added and shaken for one minute before the tubes is centrifuged and an aliquot removed. For the second dispersive SPE step the aliquot is placed in 15 mL Teflon centrifuge tubes for clean-up. The tubes already contain pre-weighted $MgSO_4$, Primary Secondary Amines (PSA) and C18 (Ramalhosa *et al.,* 2009; Pule *et al*., 2010). The composition of the extraction and dispersive steps can vary according to the method used or to the matrix of the sample to be analyzed. The methods used are either Association of Official Analytical Chemists (AOAC) method 2007.01 or European Norn (EN) method 15662 (Agilent SampliQ Recommended Standard Operating Procedure for QuEChERS). Advantages of the QuEChERS method are the pre-weighted salt and sorbents kits which allow for time saving as well as reduction in human errors. Another positive aspect is that ACN is compatible with HPLC analysis. The method appears to be very suitable for routine analysis. During sample preparation of plant material an extra step is added which involve the use of Graphitized Carbon Black (GCB). GCB has been reported in literature as a valid method to absorb chlorophyll and worked successfully at various concentrations ranging from 10 mg/mL of extract solution to 50 mg/mL for highly pigmented matrices (Stenerson *et al*., 2007). Despite this, some problems were reported with recovery during analysis on pesticides residues on spinach. The results showed that while the AOAC method (50 mg of GCB/mL of ACN extract) produced cleaner sample compared to the EN method (7.5 mg of GC/mL of ACN extract for “highly pigmented” produce), the recovery was reduced in the number of polar aromatic (or planar) pesticides (Zhao *et al., 2003)*. Due to the chemical nature of PAHs, compared to planar pesticides, similar interference can be

expected.

LLE is recommended in the analysis of water by many ISO and USEPA methods. The most common solvents used are hexane (ISO 17993:2002) (Wolska, 2008) and dichloromethane (EPA-610). They are very efficient solvents for extraction due to their affinity to PAHs chemical characteristics. Another advantage is the simplicity of the method and the ready availability of such solvents in a lab environment. The volumes of waste material needs to be considered when apply this technique. In this respect, Liquid Liquid Micro Extraction (LLME) could be a valuable alternative, since the solvents for extraction are used in smaller volumes, but problems can be encountered when working with compounds present in the environment at trace levels.

#### 2.2.3.7 Chromatography and detection – High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC)

The International Organization for Standardization (ISO), as well as US EPA, have set standard methods for determination of PAHs in environmental media and tables with a summary of the specific codes are given in Poster *et al.* (2006). HPLC-FLD and GC-MS are both widely recommended standard methods and the coupling of both gives the possibility of measuring a wide range of PAHs.

HPLC-FLD has the capacity to analyze low concentrations of compounds of interest due to the sensitivity and the selectivity of the detection. Benzo[a]pyrene, used as a marker in EU regulations, is often analyzed with this instrument (USEPA Method 8310) (Purcaro *et al.,* 2008). Furthermore the sensitivity of the detector allows for the measurement of larger PAHs compounds, present in the environment at trace levels. Variations in the methods employed are the wavelength settings: some studies employed fluorescence detectors, set at optimized excitation and emission wavelengths, in order to obtain the best selectivity and sensitivity (Moret *et al*., 2001) and, as shown in Table 6, best results are achieved using programmable FLDs since the excitation and emission wavelengths differ between PAHs (Purcaro *et al*., 2008; Moret and Conte, 2002).

**Table 2.8** Wavelength changes for different PAHs (adapted from Moret *et.al.,* 2002)

| PAHs | λ Ex (nm) | λ Em (nm) |
|---|---|---|
| Naphthalene | 276 | 330 |
| Acenaphthene | 276 | 330 |
| Fluorene | 276 | 330 |
| Phenanthrene | 250 | 366 |
| Anthracene | 250 | 402 |
| Fluoranthene | 270 | 470 |
| Pyrene | 240 | 386 |
| Benzo[a]anthracene | 270 | 390 |
| Chrysene | 270 | 390 |
| Benzo[b]fluoranthene | 260 | 430 |
| Benzo[k]fluoranthene | 256 | 410 |
| Benzo[a]pyrene | 256 | 410 |
| Dibenzo[a,h]anthracene | 290 | 410 |
| Benzo[g,h,i]perylene | 290 | 410 |
| Indeno[1,2,3-cd]pyrene | 290 | 484 |

PAHs possess very characteristic and unique ultraviolet (UV) spectra; they are available in various databases and enable identification of individual chemicals (*e.g.* http://webbook.nist.gov). UV Diode Array Detector (DAD) is used as a tool during method development to identify single PAHs. UV-DAD can also be employed as detector for quantitation, but it does not usually possess the same sensitivity as FLD.

GC-MS is a good tool for separation, identification and quantitation. Sensitivity of GC is similar to HPLC-FLD and it is recommended as standard method in the analysis of soil, air and solid waste (ISO 18287:2006; ISO 12884:2000(E); EPA 8270C). As discussed by Poster *et al.* (2006), GC-MS provides satisfactory results in respect of the EPA 16PAHs of interest and has the ability of quantitate and identify the analytes in one method. It is the method of choice when analysing PAHs with low fluorescence like benzo[*g,h,i*]perylene and has the capacity to analyze complex matrices with hundreds of compounds.

### 2.2.4 Sensory evaluation and analysis

An accurate sensorial analysis of the experimental wines is of the outmost importance, since off-flavour in wine is the main aim of the present research. Various techniques are available to assess the organoleptic properties of foodstuff. Descriptive analysis has been identified as the most suited method due to its relevance and applicability to the study.

Descriptive analysis is a technique widely used by sensory scientists due to its refined design which involves detection and description of food visual and sensorial characteristics. Those features are subsequently qualified and quantified by a trained panel, responsible also to generate the attributes used in the analysis. The final profile of the product is described in respect to aroma, flavour, aftertaste, texture and appearance (Murray, Delahunty, & Baxter, 2001). It is an effective tool and can be used for quality control, changes in a specific product over time to determine shelf life and can establish a relationship between descriptive analysis and consumer preference.

The selection of a panel for descriptive analysis is extremely important; it is good practice to test three times more judges than the amount required for the project. The panellists should be screened for factors such as reasonable level of sensory ability, dietary habits, absence of smoking habit, use of specific medications, allergies, but one of the most important characteristics is the commitment and reliability of a person (Piggott & Hunter, 1999).

The panel of judges begins training by generating the descriptors which best describe the product (wine in this case). An experienced panel leader can help in this process since the aim of the training is to create a jargon understood and shared by every member of the panel. Standards reflecting the attributes are generally prepared to facilitate the process. The standards used to achieve consensus can be chosen from various sources, not only food-related (Rainey, 1986). The final list of attributes should be comprehensive to define the differences of the various wines, but not so extensive as to create confusion.

The following step in Descriptive Analysis will be to get the panellists to understand the intensity scale of each attribute. It is important that the judges evaluate the wine in relation with what they have experienced during training and not as personal knowledge acquired outside the panel environment. An unstructured line scale is used to quantify the attributes with regard to their intensity; this is done to avoid bias in their judgement.

The design associated with descriptive analysis is based on blind multiple tastings (replicates) of the various products and the statistical analysis carried out using Analysis of Variance (ANOVA).

## 2.3 CONCLUSIONS

Polycyclic aromatic hydrocarbons have been subject to extensive investigation for the past sixty years. They are becoming more and more cause of concern due to the exponential growth of the population and consequent industrialization experienced in the past few decades. Many countries are monitoring the levels of contamination in the environment and

legislations have been implemented to ensure that PAHs in foodstuff and drinking water do not exceed dangerous limits. To guarantee a rapid and efficient inspection of the trading goods by the competent authorities, it is necessary to develop methods which can be fast, reliable and sensitive to be implemented in routine analysis.

Progress has been made in this regard due to the development of more sophisticated instruments. Results demonstrate that both HPLC-FLD and GC-MS fulfil the regulation requirements in respect to their sensitivity and repeatability. Each of them covers a specific range of analytes: HPLC-FLD is more suited for large PAHs or those with higher fluorescence properties; GC-MS has the advantage to summarise in one method quantitation and identification, but proven to be not as sensitive in the case of large PAHs present in the environment in trace amounts.

The range of standardized methods available offers a variety of sample preparation techniques, but they focus mainly on the sample material specified by legislation namely air, water, soil, solid waste. Many articles have been also published on sample preparation for organic material such as olive oil and fish, but optimization of the procedure for other foodstuff seems it is still to be achieved. The presence of high amount of chlorophyll and colour compounds (*e.g.* anthocyanins) in some fruit and vegetables create problems with HPLC columns performance and their removal from the matrix causes the loss of some of the analytes of interest. Further research needs to be done on sample preparation for a wider range of food products. Fast and reliable techniques will provide the authorities with routine analysis able to measure and monitor PAHs in the environment and food, ensuring products with low contaminant levels and safe for human consumption.

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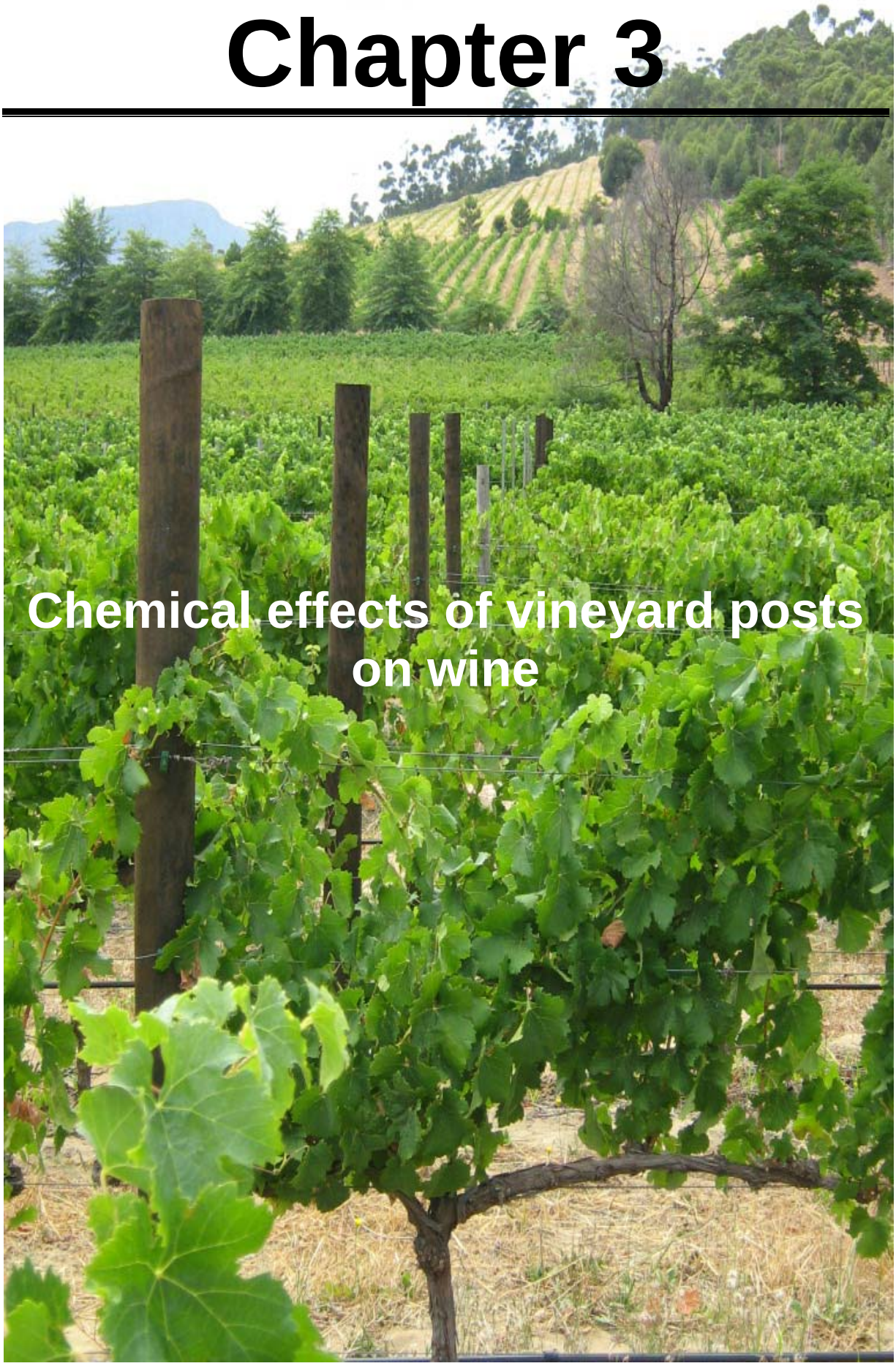
# Chapter 3



## Chemical effects of vineyard posts on wine

# 3. CHEMICAL EFFECTS OF VINEYARD POSTS ON WINE

## 3.1 INTRODUCTION

In the timber treatment industry, coal tar creosote has been for decades the leading chemical of choice for preservation of utility posts against biological and climatic decay. This preservation product has a composition which can vary according to the method of distillation used and the origin of the coal (Malber, 2004). Creosote is characterized by a smoky, medicinal and phenolic odour of very sharp and high intensity; the strong smell derives from some of the compounds among the hundreds of which creosote is made of. The numerous chemicals in the mixture can be separated into six classes: aromatic hydrocarbons, tar acids/phenolics, tar bases/nitrogen containing heterocycles, aromatic amines, sulphur containing heterocycles and oxygen-containing heterocycles (Choudhary *et al*., 2002).

Aromatic hydrocarbons include polycyclic (or poly-nuclear) aromatic hydrocarbons (PAHs) and can constitute up to 90% of the creosote mixture by weight (Melber *et al.,* 2004). They consist of fused aromatic rings: PAHs containing up to 6 aromatic rings are called “light”, while those with higher number of rings are called “heavy” PAHS. Figure 3.1 illustrate the benzene ring which is the building block of PAH compounds; naphthalene is the most volatile compound of the group (Figure 3.2), while benzo[a]pyrene (Figure 3.3) has been indicated in legislations as a marker for the monitoring of PAHs in foodstuffs (The Commission of the European Communities 2006). In the past decades this group of compounds has been the subject of intense investigation due to the fact that some of them have been proved to be toxic and carcinogenic (USDHTP, 2002).

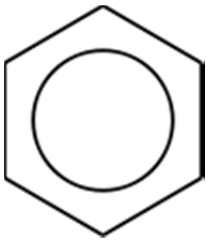


**Figure 3.1** Benzene ring ($C_6H_6$)

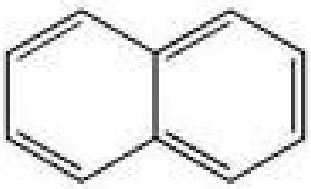


**Figure. 3.2** Naphthalene

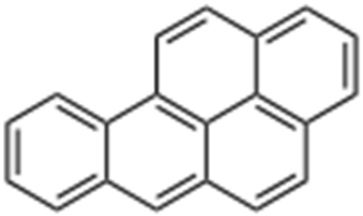


**Figure. 3.3** Benzo[a]pyrene

Apart from creosote, alternative sources of PAHs have been identified in bush fire smoke (Radojevic, 2003), grilling or smoking of food product (JECFA, 2005), burning of fuels and tobacco (Bedient, 2004). Due to the variety of contamination sources, PAHs can be found in the environment (air, water and soil), as well as in fish, fruit and vegetables which came into contact with such polluted sites (Bocca *et al.*, 2003).

A number of studies examined the uptake of PAHs by plants. Bohme *et al.* (1999) investigated the accumulation of certain PAH compounds in sunflowers and maize and linked it to particles deposition on the leaf surface. Fismes *et al.* (2002) studied the preferred route of uptake in carrots, potatoes and lettuce. They found that low molecular weight PAHs in the atmosphere are taken up by the plant *via* the leaves, while the chosen pathway for high molecular PAHs (*e.g.* BaP) is the root system. The leaves pathway is also confirmed by Kipopoulou *et al.* (1999), which indicate gaseous phase uptake *via* stomata. The same authors also suggest absorption into the waxy cuticle layer of leaves as an alternative route, based on the lipophilic nature of PAHs (Kipopoulou *et al.,* 1999).

Creosote contains also phenols in the mixture. This class includes cresols and xylenols and, despite not being present in significant amounts (less than 10%), they are found in larger percentage in the environment. Phenols water solubility allows for their wide distribution in contaminated waterways and soils (Choudhary, *et al.,* 2002). Furthermore, cresols, xylenols and phenols were among the first 12 components found in creosote vapours (Melbert, 2004), and it seems likely that they would be easily volatilized from sources, particularly under warm conditions.

South African regulations allow for the use of creosoted utility poles in agricultural land as long as they comply to the South African Bureau of Standards (SABS 457,1994), but in Europe and USA their usage has been banned (Commission Directive 2001/90/EC; Dickey, 2003).

The present study aims to determine if vines grown in a vineyard, trellised with creosoted posts, could accumulate or absorb the various malodorous compounds on the waxy layer of the berries leading to extraction of these compounds into the wine during alcoholic fermentation, creating quality and sensory problems. To establish if compounds were extracted into experimental wines, the samples were analyzed with two different methods: head space SPME in conjunction with GC-MS, Liquid Liquid Extraction (LLE) in combination with HPLC-DAD. For this purpose, methods needed to be developed for the required applications: volatile phenols methods for sample preparation and GC-MS were developed in collaboration with the Central Analytical Facility (CAF) of the University of Stellenbosch; PAHs methods for sample preparation and HPLC-DAD analysis of wine were developed by the Department of Viticulture and Oenology at the same University.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Vineyard layout

The group of projects associated with PAH investigation, and of which the present study is part, it is covered by confidentiality. Therefore the experimental block chosen for trellis system manipulation is located in the Franschhoek Valley, on a small-holding privately owned and not commercially active. The small vineyard, of approximately 1.2 hectares, was planted in 1999 with Sauvignon blanc and Merlot noir and is positioned away from main roads. Groups of 4 posts, located close to each other in parallel rows, were replaced with either metal Ecostake® poles, new certified SABS 457 creosoted poles or High Density PolyEthylene (HDPE) poles (the latter treatment only for the merlot block and growing season 2011/2012). Groups of 4 existing creosoted posts were also included in the design to represent the aging differences of the preservative material studied (see Appendix A for experimental layout). After careful consideration of soil and slope, the treatment blocks were randomly placed in the area available. Buffer areas were also considered during the treatment design, to limit the influence of the possible volatile compounds on each other. The treatment was repeated for Sauvignon blanc and Merlot noir vines. The different treatments and abbreviations used in this thesis can be seen in Table 3.1.

**Table 3.1.** Treatments and respective codes used in the experiment for both Sauvignon blanc and Merlot.

| Cultivar | Treatment | Abbreviation |
|---|---|---|
| Sauvignon blanc | Metal Poles | E |
| | New Creosoted poles | T1 |
| | Old Creosoted poles | T2 |
| Merlot | Metal Poles | E |
| | New Creosoted poles | T1 |
| | Old Creosoted poles | T2 |
| | HDPE | P |

### 3.2.2 Winemaking

Grapes for winemaking were harvested from four vines adjacent each post, two left and two on the right side, giving a total of sixteen vines per treatment, per replicate. Harvesting date was established based on sugar, titratable acidity and pH analyses as well as visual

inspection of seed maturity, petiole ripeness and general condition of the vine. Weather forecasts and climatic situation close to harvest date was also considered.

The grapes were vinified according to standard practices. Sauvignon blanc grapes were crushed, de-stemmed and a Lafazym Press® Vin blanc by Laffort (Bordeaux, France) was used as enzymatic addition at 5 g/ 100kg dosage. Bisulphite ($SO_2$) was also added at crushing at 30 parts per million (mg/L). The grapes were left to macerate for 3 hours before pressing. The pressed juice was placed at 4°C for settling overnight, then racked and inoculated with VIN 13® by Anchor Yeast (Johannesburg, SA). Fermentation occurred in glass containers in 15°C cold room and was monitored daily (Fig.3.4). At the end of fermentation the wine was racked off the lees and placed at -4°C for cold stabilization. At all times, the level of free $SO_2$ was kept at 30 ppm and the wine constantly protected from oxidation by a blanket of nitrogen gas (Afrox, SA). The wine was bottled unfiltered, in iodine sterilized bottles, capped with screw caps and placed in the storage room at a constant temperature of 15°C. The same winemaking procedure was maintained for the 3 vintages namely 2010, 2011 and 2012.

**Figure 3.4.** Sauvignon blanc glass fermenters.

**Figure 3.5.** Merlot fermentation jars.

Merlot grapes were crushed, de-stemmed and Lafase® HE Grand Cru by Laffort was used as enzymatic addition at 3 g/ 100kg dosage. Bisulphite ($SO_2$) was also added at crushing at 30 parts per million (mg/L). The grapes were left to macerate in glass jars for 5 days at -4°C: the mash was punched down once a day and protected from oxidation by carbon dioxide gas addition (Afrox, SA). After pressing, the juice was inoculated with WE372® by Anchor. Fermentation occurred in glass containers in 20°C storage room (Fig. 3.5). Punched down was carried out twice a day as well as fermentation monitoring. At the end of fermentation the wine was left to soak for further 5 days, ensuring protection from oxygen. After pressing, the wine was left to settle and then racked off the lees and placed at

-4°C for cold stabilization. At all times, the level of free $SO_2$ was kept at 30 ppm and the wine constantly protected from oxidation by a blanket of carbon dioxide gas. The wine was bottled unfiltered, in iodine sterilized bottles, capped with screw caps and placed in the storage room at a constant temperature of 15°C. The same winemaking procedure was maintained for the 3 vintages namely 2010, 2011 and 2012. After bottling routine analyses were performed to quantify pH, volatile acidity, titratable acid, malic acid, glucose, fructose and ethanol. The results are given in Appendix B.

### 3.2.3 Gas Chromatographic analysis

#### 3.2.3.1 Sampling procedure and preparation

Wine samples were taken from the bottled wines on the day of sample preparation. This was done by transferring 5 mL of wine into 20 mL SPME glass vials (Gerstel, Mülheim an der Ruhr, GE), containing 1.5 g of sodium chloride (Merck, GE) and sealed with PTFE crimp caps (Gerstel, Mülheim an der Ruhr, GE). 3-octanol (Sigma, St. Luis, MO) was used as internal standard and added to the vial at a concentration of 0.1 mg/L. The samples were stirred for 10 seconds using a vortex (Scientific Industries Inc., NY, USA) and immediately placed on the auto-sampler for analysis by GC-MS.

#### 3.2.3.2 Volatile phenols analysis

Volatile phenol concentrations in the experimental wines were analysed by GC-MS by the Central Analytical Facility (CAF) of the University of Stellenbosch (South Africa). Phenols, cresols, xylenols as well as naphthalene (Nap), benzothiophene (BTP) and acenaphthene (Ace) were quantified by a method developed by the above mentioned laboratory in collaboration with the Department of Oenology of the Stellenbosch University. The compounds were identified by mass spectra (reference standards and NIST05 spectral library collection) and quantified using ion monitoring acquired in SIM mode (Table 3.2).

The volatile compounds were extracted from the headspace of the vials by SPME method. The vials were incubated in the auto-sampler incubator for 1 minute at 50°C and subsequently a polydimethylsiloxane/divinylbenzene coated fibre was exposed to the headspace of the vial for 20 minutes at 50°C. Following this, desorption of the compounds of interest from the fibre took place in the injection port of the GC for 1 minute in splitless mode. Separation of the compounds was performed on an Agilent GC, model 6890 N (Agilent, Palo

Alto, CA), coupled with an Agilent mass spectrometer detector, model 5975 MS (Agilent, Palo Alto, CA). The system was fitted with a capillary column (60 m; 250 mm; 0.5 µm film thickness). Helium was used as carrier gas at 1.0 mL/min constant flow rate and the oven temperature programmed and ramped as follows: 40°C for 3 minutes, 100°C for 2 minutes, 180°C for 5 minutes and 240°C for 15 minutes. The total run time of the method was 44 minutes. The MSD was set for acquisition in full scan mode as well as Single Ion Monitoring (SIM).

**Table 3.2.** Volatile compounds quantified according to CAF method, their retention time (RT), monitored ions and quantifying ion (QI).

| Compound | RT (min) | Monitored Ions | | | QI |
|---|---|---|---|---|---|
| 3 Octanol (IS) | 21.50 | 101 | 129 | - | 101 |
| Naphthalene | 27.70 | 128 | - | - | 128 |
| Benzothiophene | 28.50 | 134 | - | - | 134 |
| 2,6 xylenol | 29.35 | 107 | - | - | 107 |
| o-cresol | 30.41 | 107 | 108 | 122 | 108 |
| Phenol | 30.49 | 66 | 94 | - | 94 |
| 4 Ethyl Guaiacol | 30.80 | 152 | 137 | - | 152 |
| p-cresol | 31.42 | 107 | 108 | 122 | 108 |
| m-cresol | 31.52 | 108 | 107 | - | 108 |
| 2,3 xylenol | 31.80 | 107 | 122 | - | 122 |
| 4 ethyl phenol | 32.50 | 107 | 122 | - | 122 |
| Acenaphthene | 32.90 | 154 | - | - | 154 |
| 3,4 xylenol | 33.20 | 107 | 122 | - | 122 |

### 3.2.3.3 Results and discussion

Volatile compounds found in the experimental wines varied according to vintages in both Sauvignon blanc and Merlot, but differences were not significant between treatments. In Table 3.3 concentrations of volatile compounds found in the Sauvignon blanc wines are given. Compounds present in the experimental Sauvignon blanc wines were naphthalene, 4 EP, 3,4 xylenol o and p-cresol, phenol and 4 ethyl guaiacol.

***Naphthalene***

Levels of naphthalene in the experimental Sauvignon blanc wines, ranged between 5.33 µg/L to 7.74 µg/L (Table 3.3). No significant difference were found in the levels of naphthalene between metal and old creosote treatments, while significant differences were found between the above mention treatments compare to new creosoted poles treatment

(Figure 3.6). Naphthalene concentrations were found higher in wine made from grapes grown around older creosoted posts, as an average of the three vintages (Figure 3.7), but climatic conditions have been shown to have noticeable influence on the season variation of naphthalene more than the treatments themselves. According to climatic data, registered from a nearby weather station (Agro climatology 2012), the 2010 and 2011 growing seasons have been reported as warm for the Franschhoek area, with frequent heat waves reaching up to 42°C. In contrast, the 2012 growing season was characterized by lower temperatures, cooler conditions and frequent rainfalls. These records could explain the significant lower levels of naphthalene in the 2012 vintage wines, as volatiles would not have been as readily released from the wood under cooler conditions. The drop in concentrations could also be explained by weathering of the posts, and consequent decrease of the volatile content of the poles wood preservatives.

**Table 3.3.** Concentration (µg/L)* of volatile compounds found in experimental Sauvignon blanc wines.

| Treatment | Vintage | Naph | 2,6 Xyl | 4 EP | 3,4 Xyl | o-Cr | p-Cr | m-Cr | 2,3 Xyl | Phenol | 4 EG |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Metal | 2010 | 6.76 | nd | 1.13 | 27.37 | 7.31 | 5.24 | nd | nd | 91.14 | 4.16 |
| Old Creo | 2010 | 7.28 | nd | 3.49 | 26.97 | 21.06 | 3.86 | nd | nd | 82.54 | 3.72 |
| New Creo | 2010 | 6.48 | nd | 2.28 | 29.10 | 3.83 | 5.21 | nd | nd | 79.16 | 4.15 |
| Metal | 2011 | 7.74 | nd | nd | 29.45 | 15.98 | nd | nd | nd | 74.92 | 5.40 |
| Old Creo | 2011 | 8.28 | nd | nd | 25.01 | 8.29 | 1.34 | nd | nd | 71.75 | 5.37 |
| New Creo | 2011 | 7.38 | nd | nd | 31.31 | 13.18 | nd | nd | nd | 81.38 | 6.41 |
| Metal | 2012 | 5.33 | nd | nd | 33.63 | nd | nd | nd | nd | 115.95 | nd |
| Old Creo | 2012 | 5.37 | nd | nd | 28.36 | nd | nd | nd | nd | 112.02 | nd |
| New Creo | 2012 | 5.39 | nd | nd | 28.70 | nd | nd | nd | nd | 112.14 | nd |

(*) Values are means of five replicate analyses.

nd = not detected.

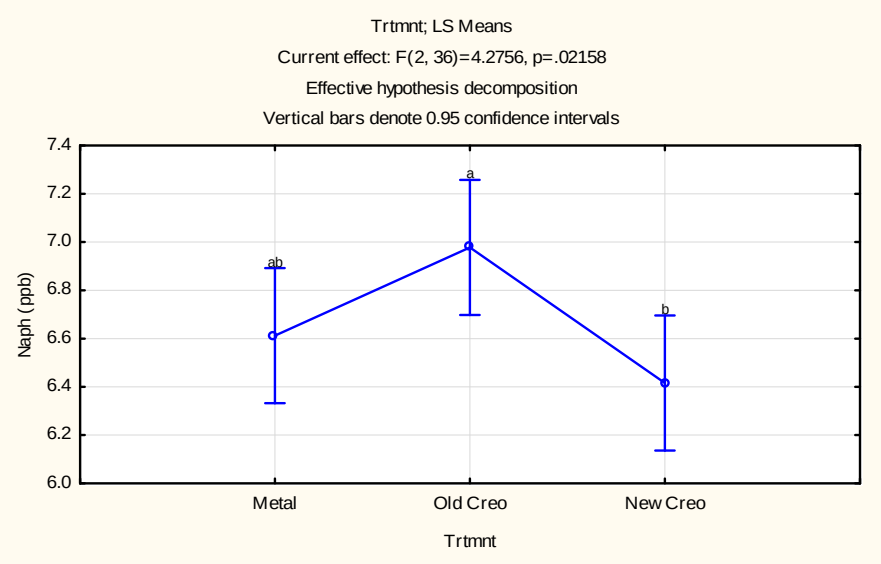



**Figure 3.6.** Naphthalene concentrations (µg/L) found in Sauvignon blanc wines produced from the 'metal', 'old creosoted' and 'new creosoted' treatments. The values indicate means of the treatment replicates as well as the three vintages for the same treatment. Error bars indicate 95% confidence intervals for the means and the letters indicate differences on a 5% significance level.

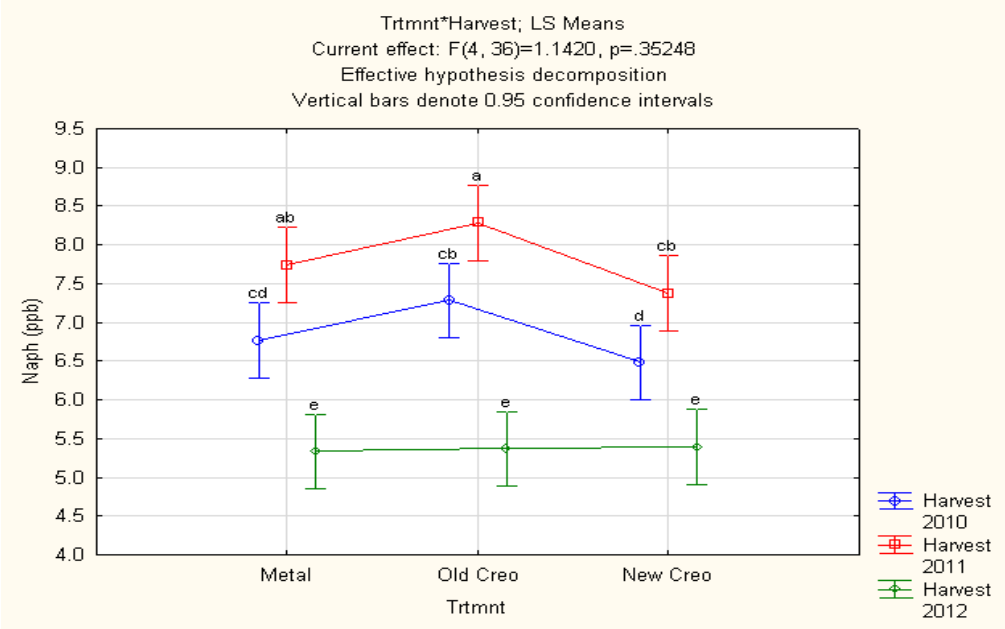



**Figure 3.7.** Naphthalene concentrations (µg/L) found in Sauvignon blanc wines for the 2010, 2011 and 2012 harvests, produced from the 'metal', 'old creosoted' and 'new creosoted' treatments. The values indicate value means of the treatments.

Levels of naphthalene in the experimental Merlot wines, ranged between 5.17 µg/L and 12.27 µg/L (Table 3.4). No significant differences were found in the levels of naphthalene between treatments, while significant difference was found between harvest seasons (Figure 3.8).

**Table 3.4.** Concentration (µg/L)* of volatile compounds found in experimental Merlot wines.

| Treatment | Vintage | Naph | 2,6 Xyl | 4 EP | 3,4 Xyl | o-Cr | p-Cr | m-Cr | 2,3 Xyl | Phenol | 4 EG |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Metal | 2010 | 10.92 | nd | nd | 37.94 | 6.99 | 1.47 | 1.16 | nd | 86.03 | 13.78 |
| Old Creo | 2010 | 12.27 | nd | 17.94 | 35.88 | 11.18 | 2.74 | 3.66 | 4.83 | 90.56 | 10.19 |
| New Creo | 2010 | 8.94 | nd | 15.30 | 46.27 | 16.86 | 4.70 | 3.01 | nd | 99.75 | 9.26 |
| Metal | 2011 | 6.33 | nd | 33.75 | 24.14 | 2.84 | 4.62 | 1.14 | 13.51 | 98.67 | 0.91 |
| Old Creo | 2011 | 5.39 | nd | nd | 43.52 | 9.81 | 1.73 | nd | nd | 75.55 | 0.72 |
| New Creo | 2011 | 5.98 | nd | 21.42 | 41.05 | 7.69 | 4.70 | 6.36 | 3.56 | 98.96 | 1.05 |
| Metal | 2012 | 5.23 | nd | nd | 37.14 | nd | nd | nd | nd | 98.50 | nd |
| Old Creo | 2012 | 5.22 | nd | nd | 39.81 | nd | nd | nd | nd | 86.10 | nd |
| New Creo | 2012 | 5.63 | nd | 11.03 | 37.13 | 4.07 | 2.21 | 1.50 | 3.41 | 91.56 | 0.54 |
| Plastic | 2012 | 5.17 | nd | nd | 24.98 | nd | nd | nd | nd | 74.19 | nd |

(*) Values are means of five replicate analyses.

nd = not detected.

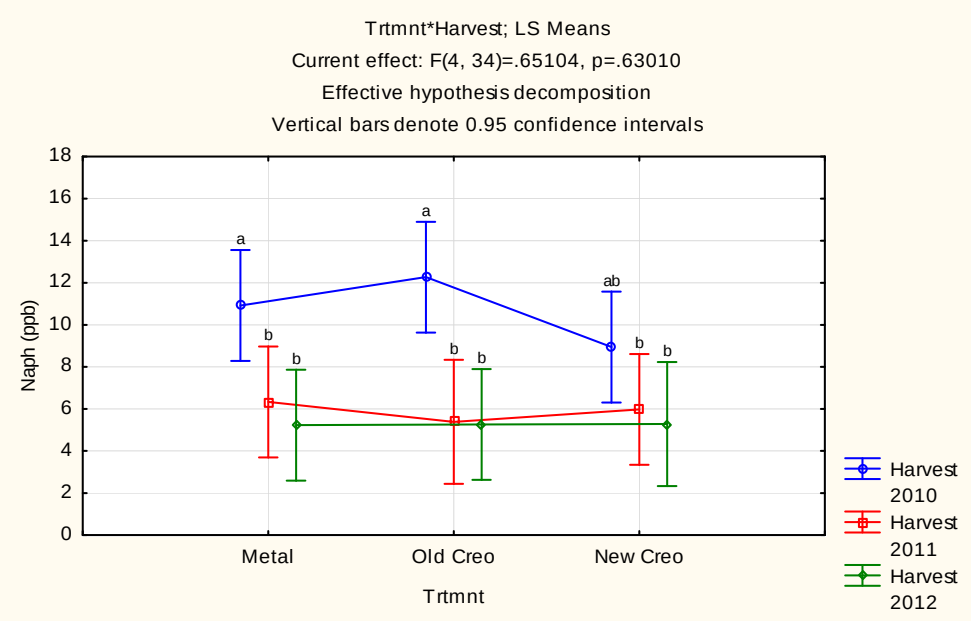



**Figure 3.8** Naphthalene concentrations (µg/L) found in Merlot noir wines for the 2010, 2011 and 2012 harvests, produced from the 'metal', 'old creosoted' and 'new creosoted' treatments. The values indicate means of the treatments.

Levels of naphthalene for the 2010 vintage are noticeably higher for Merlot compared to Sauvignon blanc concentrations, while similar concentrations are observed for the other two vintages. Those results could be explained by the amount of days of exposure of the grapes

to the different treatment: replacement of the posts for the first growing season happened in January 2010 and Sauvignon blanc grapes were harvested on the 2nd of February, exactly a month after the beginning of the trial. Merlot grapes were harvested on the 4th of March, allowing for an extra month of exposure of the berries to volatiles compounds. In the following two vintages, the concentrations of naphthalene for both cultivars were very similar and the time of exposure to the volatile compounds was the same. A rapid decrease in naphthalene concentration in the second harvest can easily be explained by the physical characteristics of the compound. Naphthalene is an extremely volatile compound which volatilises at room temperature (International Programme on Chemical safety *n.d.*). It can constitute 3 to 18% by weight of the creosote mixture (Melber 2004). Nevertheless the concentrations seem to stabilize after the first year of weathering. The lack of difference between treatments could be explained by the variety of sources for naphthalene contamination, which include cigarette smoke, exhausts from motor vehicle traffic and smoke from forest fires (Radojevic 2003). The latter one, in particular, is a reoccurring problem for the Western Cape region. Exposure of grapevines to the treatments in a controlled environment would have been ideal to avoid interferences in the system, but logistics reasons, as well as the willingness to conduct a study on fully grown vines, representative of a commercial situation, dictated the choice of a field trial.

Naphthalene content in foodstuff has been reported in carrots, cabbage and leeks grown on a contaminated soil close to an industrial area. The vegetables had naphthalene concentrations ranging from 0.37 to 63 µg/kg dry weight (Kipopoulou, Manoli and Samara 1999). Higher concentration has been reported in shellfish between 5 to 176 µg/kg (International Agency for Research on Cancer 2002). Regulatory guidelines are set at 0.1 mg/kg/day as chronic reference dose (RfD) by USEPA, where "the RfD is an estimate of the quantity of chemical that a person could be exposed to every day for the rest of their life with no appreciable risk of adverse health effects" (Gervais, et al. 2010). In light of these guidelines, the amounts detected in the experimental wines are largely below the safe limits.

***Other volatile phenols***

Other volatile phenols identified and detected during GC analysis were 4-ethylphenol, 3,4-xylenol, o-, p- and m-cresol, 2,3-xylenol and phenol (Tables 3.3 and 3.4). None of the compounds were found to be significantly different in relation to the treatments, but they also appear to be decreasing with the same trend as naphthalene, probably due to the climatic data, as explained above, or the weathering of the poles exposed to the environment. Odour threshold for 4-ethylphenol, 3,4-xylenol, m-cresol and phenol are 605 µg/L, 1200 µg/L, 68 µg/L and 7100 µg/L, respectively. All the amounts found in wine were below the odour threshold of the corresponding compounds.

The lack of significant differences between treatments could be due to the experimental layout, as explained per naphthalene. It is possible that if an entire block of vineyards was planted with creosoted posts, the cumulative effect may produce different results. A parallel study, started in the 2011/2012 growing season, is monitoring the effect on grapes and wines produced from a vineyard planted with a creosoted pole for each vine. The results of this experiment, due by the end of 2013, could expand the findings on the subject.

### 3.2.4 HPLC analysis for PAHs

In order to test the hypothesis that PAHs could be transferred from the creosoted posts to the waxy layer of the grape berries, or taken up by the roots, and later ending up in the wine, methods needed to be developed. A sample preparation procedure, as well as an instrumental method for HPLC-DAD, was therefore developed, as part of this study.

#### 3.2.4.1 Sampling preparation procedure

All the glassware used during sample preparation was washed and rinsed with distilled water filtered with a Milli-Q system (Millipore, Bedford, Mass., USA) and left to dry in a forced circulation incubator (Labcon, CA) at 60°C.

Wine samples were taken from the bottled wines, stored at 15°C, on the day of preparation. Sample preparation for PAHs analysis was done by Liquid Liquid Extraction (LLE): 25 mL of wine were pipetted into 35 mL Pyrex® glass tubes; 2-ethyl-fluorene (IS) was added at a concentration of 40 µg/L; 3 mL of cyclohexane (Sigma, St. Luis, MO) were used as extraction solvent and finally, the glass tube was capped, shaken and placed in the ultra sonication bath (Branson Ultrasonic Corporation, Danbury, Connecticut, USA) for 20 minutes. Vials were shaken at 5 minutes intervals. After ultrasonication, the sample glass tubes were placed in the freezer at -20°C for 15 minutes in order to reduce internal vapour pressure. The top organic layer was removed using a glass Pasteur pipette (Stargate Scientific, Wilgeheuwel, SA), transferred into a 15 mL glass Pirex® tube and the latter closed with screw top and set aside for later addition. The extraction steps were repeated a second time with further 3 mL of cyclohexane and the organic layer combined with the organic layer already transferred to the 15 mL glass tube; the tubes were placed in the centrifuge for 15 minutes at 4000 rpm (HERMLE Labortechnik GmbH, Wehingen, Germany). Half way through the centrifugation cycle, the tubes are taken out and shaken as in random samples an emulsion forms between the layers and this step improves their separation. The clear organic layer was transferred into new 15 mL glass tubes, containing sufficient sodium sulphate anhydrous ($Na_2SO_4$, Merck, GE) to cover the bottom and placed on a Vortex (Scientific Industries Inc., NY, USA) for 30 seconds. $Na_2SO_4$ acts as absorbing salt to

remove the aqueous phase potentially transferred with the organic layer. The dried organic layer was then poured into new glass tubes, placed in a Techne, Dri-block® DB-3D sample concentrator (Techne, Staffordshire, UK) under a stream of nitrogen (Afrox, SA) at a controlled temperature of 40°C and brought to dryness. After evaporation, the residue was re-dissolved in 1 mL of acetonitrile (Merck, GE) and vortexed for 1 minute. The acetonitrile-based mixture was transferred into a 1 mL syringe (Surgi Plus, Orissa, India) fitted with a 0.45 µm nylon filter (Bonna-Agela Technologies, Wilmington, DE) and placed into an amber HPLC glass vial (Stargate Scientific, Wilgeheuwel, SA) and sealed with a silicon/PTFE septa cap. The samples were stored in a -20°C freezer until analysis.

The above sample preparation technique was chosen over the QuEChERS® method patented by Agilent Technologies (Agilent, Palo Alto, CA) for reasons which will be discussed below. The acronym QuEChERS® stands for: quick, easy, cheap, effective, rugged and safe. QuEChERS® is a method for food sample preparation for analysis on HPLC and GC which was developed by Anastassiades, *et al.*, (2003) and published in the Journal of AOAC. It was originally a non-buffered method and was subsequently it modified for extraction of pH dependant compounds. Further changes minimized the degradation of susceptible compounds and expanded the matrices covered.

The kit is divided in 2 steps each with its own pre-prepared, pre-weighted salt sachet. *Step 1* is composed of a 50 mL Falcon tube and a sachet containing magnesium sulphate ($MgSO_4$), sodium acetate according to the AOAC method. The content of the sachet might change according to the method chosen and/or according if it is buffered or not. *Step 2* is composed of a 15 mL centrifuge tube containing pre-weighted SPE sorbent and $MgSO_4$ but, also in this case, the content might vary according to the matrix. The function of the SPE sorbent is to pull out interfering matrix material (e.g. Primary Secondary Amines to remove fatty acids) while the magnesium sulphate removes excess water and improve analyte partitioning. The QuEChERS® method by Agilent Technology has been created to simplify procedures by reducing the time dedicated to sample preparation in foodstuff analysis. It addresses many needs and even if initially it was developed for routine analysis on pesticides, it is employed nowadays for extraction of veterinary drugs in animal tissues, mycotoxins in farming products, environmental compounds in soil, and new applications are under investigation. The adaptability of the method makes it possible to be used on various matrices such as grains, seeds, tobacco, coffee and beverages like milk, wine and water. The chromatogram shown in Figure 3.9 represents a comparison between LLE technique and QuEChERS® method on spiked red wine extraction. The LLE technique is displayed in red, while the QuEChERS® method in blue on the main chromatogram. It can be immediately noted that the intensity of the signal for LLE is much higher than the

QuEChERS® one, due to the higher yield of the LLE technique. This fact is extremely important for identification of compounds of interest: identification of unknown compound can be obtained comparing its UV spectrum to the software library. The higher the signal, the better the chance of a fit will be with the library corresponding compound, as illustrated in figure 3.9. Insert A and B represent the match between the spectra of chrysene from the library (red) and the spectra from the chromatogram (blue). The better fit in insert B is due to the higher signal produced by the LLE peak.

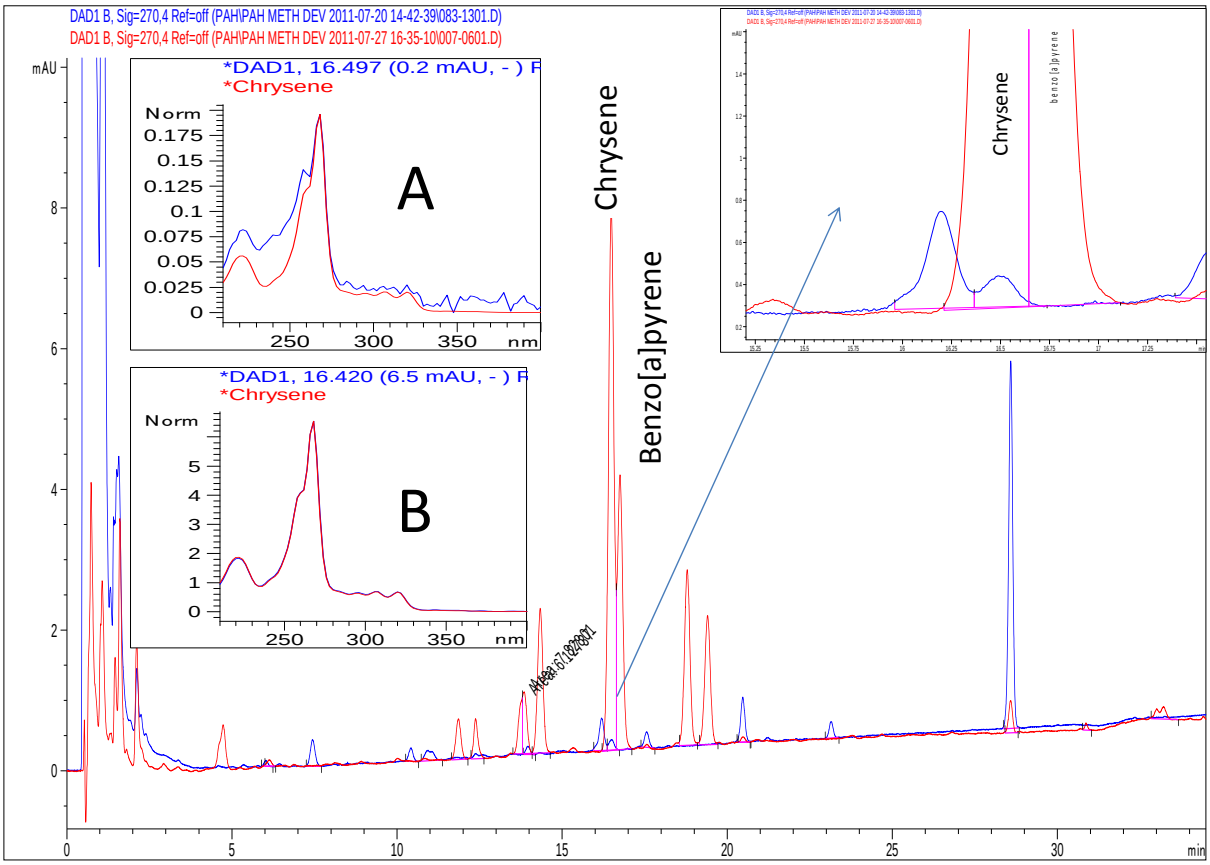



**Figure 3.9.** Chromatogram of spiked red wine extraction, comparing LLE technique (red) to QuEChERS® method (blue). The inserts on the left side of represent the comparison (match) between the UV spectra of the compound of interest in the library (red) and the spectra of the same compound extracted from the chromatogram (blue).

### 3.2.4.2 Materials

The solvents used were HPLC grade acetonitrile (Merck, GE) and distilled water filtered with a Milli-Q system (Millipore, Bedford, Mass., USA). The standard used for calibrations were benzo[a]pyrene (BaP), Pyrene (Pyr), Fluoranthene (Fla), Fluorene (Fluo), Benzo[b]fluoranthene (BbF), Acenaphthene (Ace), Acenaphthylene (Acy), Phenanthrene

(Phe) from Sima-Aldrich (Sigma, St. Luis, MO); Chrysene (Chr), benzo[a]Anthracene (BaA), Anthracene (Ant) from Fluka (Buchs, Switzerland) and Benzo[g,h,i]perylene (BghiP) from Supelco (Bellafonte, PA, USA). 2-ethyl-anthracene (Sigma, St. Luis, MO) was chosen as internal standard.

### 3.2.4.3 Chromatographic conditions

An Agilent 1260 Infinity HPLC system (Palo Alto, CA) was used for PAHs analysis. The column was a C18 reverse-phase (Poroshell® 120), 4.6 x 50 mm i.d. 2.7 micron particle size (Agilent, Palo Alto, CA) maintained at a temperature of 25°C during analysis. The instrument is equipped with ChemStation® software (Agilent, Palo Alto, CA).

The mobile phase consisted of water (A) and acetonitrile (B) at a flow rate of 1 mL/min. and programmed to reach 100% acetonitrile as follows: the mobile phase is maintained isocratic for 2 min. at 40% B; the gradient is increased to 80% B in 20 min.; brought to 100% B over a period of 4 min.; finally maintained isocratic at 100% B for 3 min. The total run time was 34.5 minutes, including reconditioning of the column.

The DAD detector was programmed to take readings from 190 to 400 nm for identification of the compounds and quantification was carried out at 230, 254, and 270 nm. The resulting chromatograms, and the compounds monitored at each wavelength, can be seen in Figure 3.10.

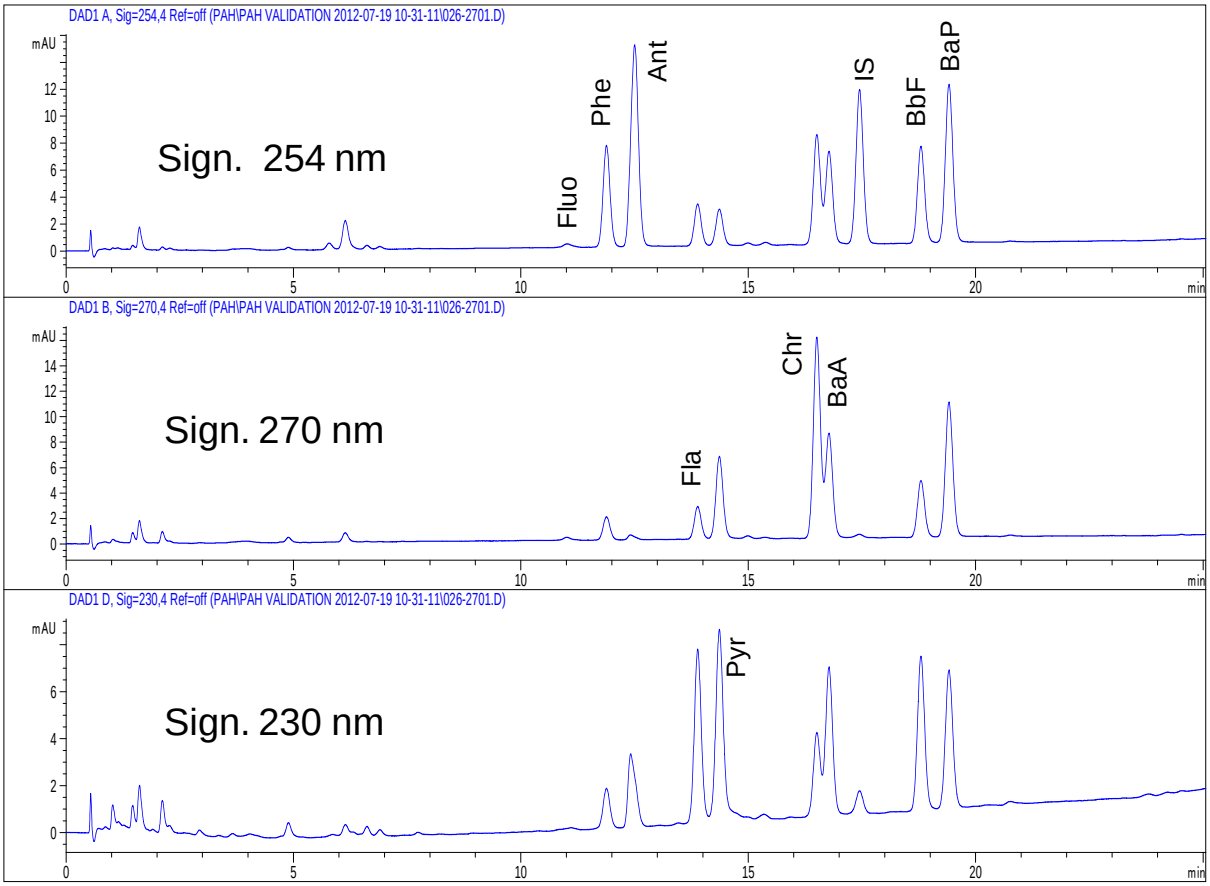



**Figure 3.10** Chromatograms of red wine spiked with 11 PAHs at 100 ppb. The wavelengths monitored were 254 nm for Fluorene (Fluo), Phenanthrene (Phe), Anthracene (Ant) Benzo[b]fluoranthene (BbF), Benzo[a]pyrene (BaP) and 2 Ethyl Anthracene (2EA) as internal standard; 270 nm for Fluoranthene (Fla), Chrysene (Chr) and Benzo[a]Anthracene (BaA); and 230 nm for Acenaphthene (Ace), Acenaphthylene (Acy) and Pyrene (Pyr).

The LLE and HPLC methods for PAHs in wine were validated and Limit of Detection (LOD), Limit of Quantitation (LOQ) and recoveries values for each compound are as shown in Table 3.5. All the recovery values are between 82% and 126%, except for the first 3 PAHs of interest. During repeatability testing acenaphthylene, acenaphthene and fluorene showed non reproducible behaviour. These results can be attributed to the semi-volatile nature of the compounds and explained by partial evaporation during the drying step of sample preparation. Due to this particular issue no values are indicated for those compounds in Table 3.5. In Figure 3.10, acenaphthylene and acenaphthene should appear in the chromatogram for the 230 nm wavelength (RT 8.88 and 10.87 min. respectively), but while

the respective peaks were sufficient for identification, they were too low for quantitation. The same problem was encountered for fluorene, retention time 11.07 minutes in the 254 nm chromatogram. The remaining PAHs were recovered in satisfactory amounts with LOD and LOQ complying with European regulations, in particular with regard to Benz[a]pyrene. Commission Directive 2005/10/EC (4$^{th}$ February 2005) regulates sampling methods and requirements for the methods of analysis for the official control of the levels of BaP in foodstuff (The Commission of the European Communities 2005). The performance criteria for methods of analysis are: LOD of at least 0.3 ppb, LOQ of at least 0.9 ppb and recoveries included between 50 and 120%. All the prescribed criteria were met by the method developed in the course of the present study.

**Table 3.5.** Performance criteria obtained for the LLE method and the HPLC-DAD instrumental method developed.

| Compound | LOD | LOQ | Recovery white wine | | Recovery red wine | |
|---|---|---|---|---|---|---|
| | ppb | ppb | 25ppb | 100ppb | 25ppb | 100ppb |
| **Acy** | | | | | | |
| **Ace** | | | | | | |
| **Fluo** | | | | | | |
| **Phe** | 0.4016 | 1.3387 | 115% | 115% | 108% | 87% |
| **Ant** | 0.1307 | 0.4355 | 87% | 100% | 82% | 86% |
| **Fla** | 1.6043 | 5.3476 | 88% | 91% | 99% | 106% |
| **Pyr** | 0.6303 | 2.1008 | 97% | 88% | 110% | 98% |
| **Chr** | 0.2650 | 0.0883 | 97% | 101% | 113% | 118% |
| **BaA** | 0.5226 | 1.7422 | 97% | 99% | 110% | 112% |
| **BbF** | 0.6237 | 2.0790 | 96% | 104% | 111% | 121% |
| **BaP** | 0.3916 | 1.3055 | 102% | 108% | 118% | 126% |

### 3.2.4.4 Results and discussion

Experimental Sauvignon blanc and Merlot wines were prepared with LLE method and analysed by HPLC-DAD. Both methods were developed by the Department of Oenology (Stellenbosch University, SA) during the course of the present study. The retention time (RT) of each compound, the wavelength used for quantitation, the calibration curve, and the range of detection analysed in wine are summarised in Table 3.6. All the compounds could be identified using the spectra extracted and retention timesbut acenaphtylene, acenaphthene and fluorene could not be quantified with sufficient accuracy as demonstrated by the poor $R^2$ values.

**Table 3.6.** Retention time (RT) of each compound, the wavelength (λ) used for quantitation, the calibration curve, and the range of detection analysed in wine before extraction.

| Compound | RT (min) | λ | Calibration curve | $R^2$ | Range in wine (µg/L) |
|---|---|---|---|---|---|
| **Acy** | 8.88 | 230 | y = 0.0003x + 0.012 | 0.9276 | |
| **Ace** | 10.87 | 230 | y = 0.0011x + 0.0215 | 0.9706 | |
| **Fluo** | 11.07 | 254 | y = 0.0024x + 0.0019 | 0.9872 | |
| **Phe** | 11.87 | 254 | y = 0.0094x – 0.0206 | 0.9989 | 10-250 |
| **Ant** | 12.49 | 254 | y = 0.0185x + 0.0765 | 0.9988 | 5-250 |
| **Fla** | 13.93 | 270 | y = 0.0021x + 0.0023 | 0.9998 | 5-250 |
| **Pyr** | 14.38 | 230 | y = 0.0065x + 0.0014 | 0.9999 | 5-250 |
| **Chr** | 16.50 | 270 | y = 0.0108x + 0.0186 | 1 | 5-250 |
| **BaA** | 16.77 | 270 | y = 0.0061x + 0.0012 | 1 | 5-250 |
| **BbFl** | 18.79 | 254 | y = 0.0049x - 0.0064 | 0.9997 | 25-250 |
| **BaP** | 19.41 | 254 | y = 0.0018x - 0.0114 | 0.9996 | 10-250 |

Sauvignon blanc wines contained only one of the PAHs of interest, namely chrysene, at levels ranging from 3.8 to 4.7 µg/L (Table 3.7). Differences between treatments were noted for the first harvest season, but no significant differences were detected in the following years (Figure 3.11).

**Table 3.7 Chrysene concentrations found in Sauvignon blanc experimental wines expressed in µg/L. The values are means of five analysis repetitions.**

| Sample | Treatment | Harvest | Chrysene |
|---|---|---|---|
| SB | Metal | 2010 | 4.7 |
| SB | Old Creo | 2010 | 3.8 |
| SB | New Creo | 2010 | 0.0 |
| SB | Metal | 2011 | 4.6 |
| SB | Old Creo | 2011 | 4.6 |
| SB | New Creo | 2011 | 4.7 |
| SB | Metal | 2012 | 4.5 |
| SB | Old Creo | 2012 | 4.5 |
| SB | New Creo | 2012 | 3.7 |

According to the Scientific Committee on Food, “BaP can be used as a marker for the occurrence and effect of carcinogenic PAHs in food including... chrysene” (Scientific Committee on Food 2002). The maximum levels allowed ranges between 5.0 µg/kg of wet weight for smoked meats to 10.0 µg/kg for bivalve molluscs (The Commission of the

European Communities 2006). In light of such regulations, the concentrations of chrysene found in the experimental wines fall within the prescribed parameters.

Since no other PAH compounds were found in the samples analysed, it can be concluded that the wine is safe for human consumption and complies with EU regulations.

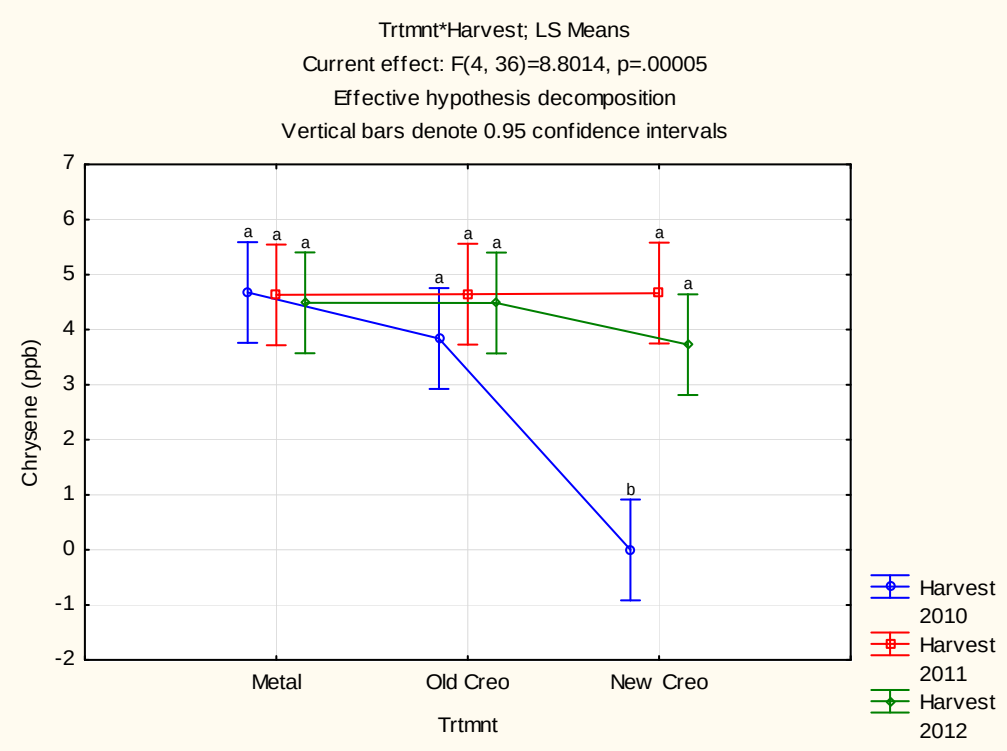



**Figure 3.11.** Chysene concentrations found in experimental Sauvignon blanc wines. Significant differences between treatments were found in the first 2010 vintage, but no differences could be observed in the following years.

None of the vineyard treatments had an influence on experimental Merlot: wines were found free of PAHs of interest. Chatonnet *et al.,* (1992), found that aging of wine on lees decreased the impact of wood taste and aroma; this effect was induced by the binding of volatile phenols to the yeast biomass. Furthermore, Lubbers *et al*., (1994) reported that a greater degree of binding was observed between volatile phenols and lees in model wine for more hydrophobic molecules (Lubbers, *et al.* 1994): the lipid content of yeast cell walls was responsible for the phenomenon. Due to skin contact applied to winemaking of Merlot, before and after fermentation, and extended lees contact of the pressed wine, it can be speculated that a similar behaviour could have induce binding of non polar PAHs to yeast biomass. An extensive investigation on this aspect of the project will be covered by a parallel study conducted by the Department of Oenology and Viticulture of Stellenbosch University.

## 3.3 CONCLUSIONS

Sauvignon blanc and merlot wines made from grapes grown in proximity of old and new creosoted posts were analysed by HPLC and GC for PAHs and volatile phenols respectively. PAHs were found only in Sauvignon blanc experimental wines, in levels below maximum allowed limits, according to European Community legislation. Volatile phenols were detected in both Sauvignon blanc and Merlot experimental wines at concentrations below odour threshold. Nevertheless, the wines produced by grapes grown next to the new creosote poles, displayed a 'burnt rubber', 'phenolic', 'smoky' and 'medicinal' characteristics when subject to sensory analysis. Absorption of compounds contained in the creosote mixture into the waxy layers of grape berries was proven for naphthalene and some of the volatile phenols. Significant difference between treatments was observed for naphthalene concentrations in Sauvignon blanc, while no differences were found in merlot wines. The discrepancy could be explained by the diverse winemaking practices followed, where the red variety is exposed to a less reductive process.

These results represent an experimental trial, limited in extension. Further investigation is recommentded to evaluate a cumulative effect of creosoted posts in a fully trellysed vineyard. Furthermore, the creosote mixture comprises of more then 200 chemical compounds and it is therefore an extremelly complex matrix. It is possible that if a greater number of PAHs and volatile phenols were to be monitored, different results could be obtained.

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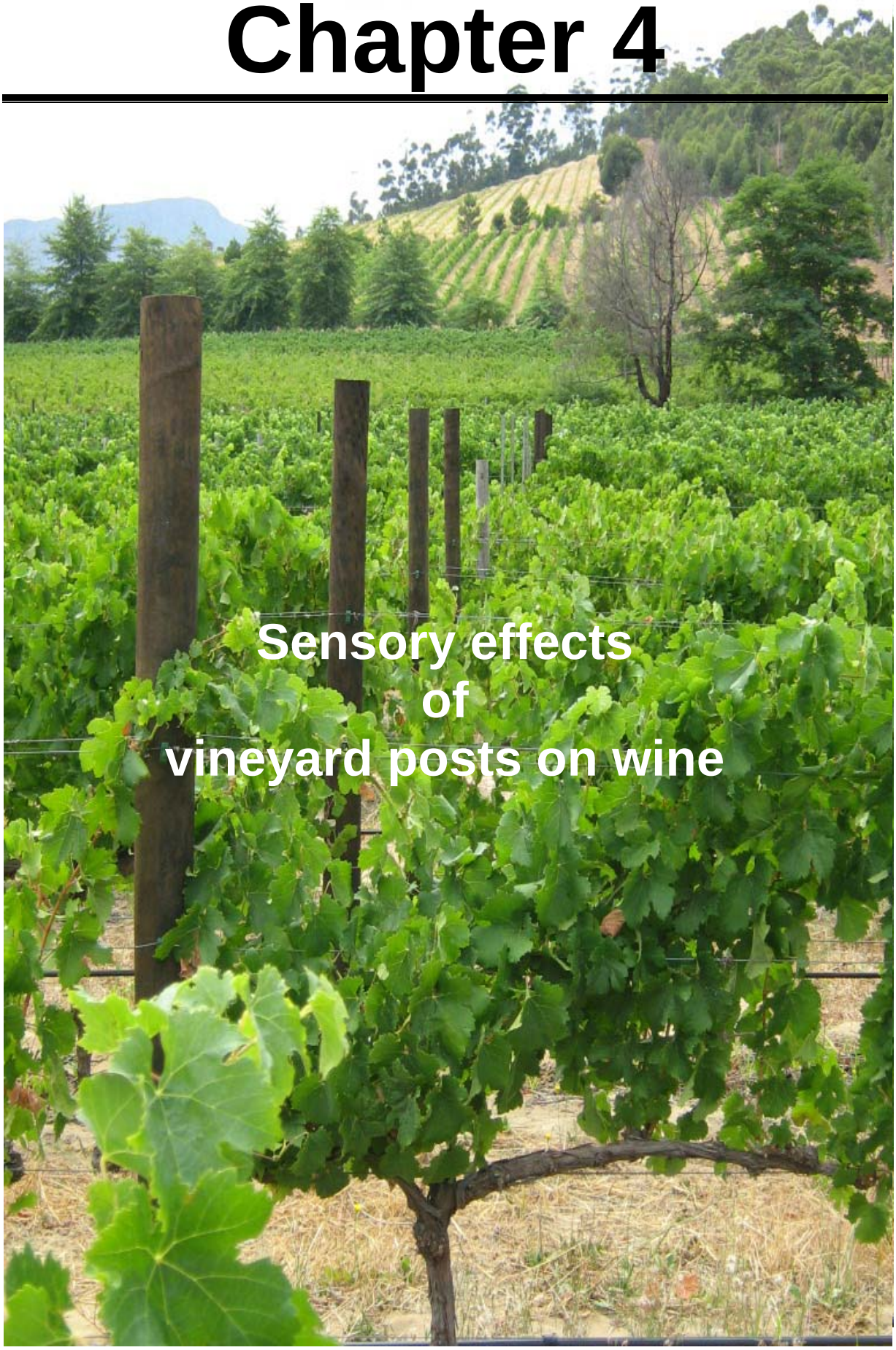
# Chapter 4



## Sensory effects of vineyard posts on wine

# 4. SENSORY EFFECTS OF VINEYARD POSTS ON WINE

## 4.1 INTRODUCTION

Volatile phenols are a class of compounds which includes many sub-groups such as ethylphenols, vinylphenols, creosols and xylenols. Many of these compounds are an integral part of the complex aroma profile of wine when present at low levels, but can represent a threat to the wine quality if present at high concentrations, as they might dominate and impart smoky, charred or burnt aromas that detract from the quality of the wine. Chatonnet *et al*. (1992) showed that volatile phenols are produced during malolactic fermentation by yeast of the genus *Brettonomyces* during the enzymic decarboxylation of cinnamic acids into volatile phenols. In addition, *p*-coumaric acid, present in grapes, is converted into 4-vinylphenol during alcoholic fermentation by *Saccaromyces cerevisiae.* A further step in the process can occur if *Brettonomyces* species are present in the wine, when 4-vinylphenol will be converted into 4-ethyl phenol by vinyl-phenol reductase (Chattonnet *et al.,* 1992).

Volatile phenols are part of the aroma and flavour profile of variety of food products including essential oils, tea, roasted coffee and wine (Merisol USA LLC 2009). Fernandez de Simon and co-workers (Fernandez de Simon, *et al*., 2008, and 2010) investigated the presence of a number of phenols in wine including phenol itself, the cresols and 4-ethyl phenol and linked their origin to oak wood pyrolysis. 3,4-xylenol (or 3,4-dimethylphenol) has also been detected in wine treated with toasted American oak (Kaushal, 2007), but in previous work by Etievant (1981), cresols and xylenols were also found in unwooded wines, demonstrating that wood treatment was not solely responsible for their occurrence in the finished product. In a review by Radojevic (2003) about the chemistry of forest fires and regional haze, the composition of wood smoke was discussed, and more than 100 compounds were identified, including volatile organic compounds such as phenols and cresols. The smoke particles were transported several kilometres away from the source and deposited on crops grown over a large area (Radojevic, 2003). Grapes and wine affected by smoke have been shown to contain volatile phenols, including o-cresol and phenol (Hayasaka *et al.*, 2010). Kennison *et al.*, (2007) demonstrated direct association between grapes exposed to smoke during the growing season and the presence of smoke related compounds in the final wine. The aromas produced were described as ‘burnt rubber’, leather’, ‘disinfectant’ and ‘smoked meat’ (Kennison, *et al*. 2011). More odour descriptors and their relative thresholds are given in Table 4.1.

**Table 4.1.** Odour thresholds (µg/L) and descriptors of some volatile phenols found in the experimental wines. 4 EP has been determined in red wine, while the other phenols in aqueous solutions with or without ethanol.

| Compound | OT (µg/L) | Odour | Reference |
|---|---|---|---|
| 4-ethylphenol | 605[1] | Wet horse, animal | Chatonnet *et al.,* 1992 |
| Phenol | 7100[2] | Artificial sweetness | HPA*<br>Parker *et al*., 2010 |
| p-cresol | 10[2]<br>3.9[3] | Chemical, tar-like, mothballs | HPA*<br>Parker *et al*., 2010 |
| o-cresol | 31[2] | Smoky, tar-like | Parker *et al*., 2010 |
| m-cresol | 68[2]<br>15[3] | Medicinal | Parker *et al*., 2010 |
| 3,4-xylenol | 1200[2] | Sick sweet | Burdock, 2010 |

(*) Health Protection Agency –Odour complaints checklist (2011)

([1]) OT in red wine

([2]) OT in aqueous solution at 10% ethanol

([3]) OT in water

Alternative sources of contamination in food products may come from motor vehicle exhausts and residential woodstove emissions in highly populated areas (Allen, 2009). A mixture of cresols, xylenols and phenols are also known in the chemical industry as cresylic acid and it is one of the components of creosote. This mixture is used in the preservation of wooden utility poles in various industries, including agriculture (Merisol, 2009). These compounds do not constitute a high percentage of the creosote mixture (approximately 5-10%), but they are more soluble in water and therefore they are found in high percentage in contaminated water, soil and sediments (Choudhary *et al.,* 2002). Cresols, xylenols and phenols were among the first 12 components found in creosote vapours (Melbert, 2004), and it seems likely that they would be easily volatilized from sources, particularly under warm conditions.

The following results explain the effects of new and old creosote poles on the organoleptic properties of Sauvignon blanc and Merlot experimental wines. An additional study examined the interaction of phenol, o-cresol, 4 ethyl phenol and 3,4-xylenol at 4 different concentrations in a control red wine, to determine whether a synergistic effect between them exists. Interaction between the phenols could intensify the taint in wine even at individual levels below detection threshold.

## 4.2 MATERIALS AND METHODS – CREOSOTE TRIAL

### 4.2.1 Experimental design and winemaking

Vineyards layout, experimental design and winemaking techniques are described in Chapter 3, section 3.2.1 and 3.2.2.

### 4.2.2 Sensory evaluation of wines: training of the panel and tasting technique

Descriptive analysis was employed to investigate the aroma characteristics of the experimental wines and to identify possible taints. A panel of 12 judges was selected for the sensory analysis, all with moderate to good experience in wine evaluation and working for the Department of Oenology of the Stellenbosch University, either as academics or postgraduate students. The sensory analyses were performed after approximately 5 months from bottling date and therefore the composition of the panel varied slightly from year to year. The panel of judges began training by familiarising themselves with the compounds of interest. This was achieved by spiking a neutral wine with 4-ethylphenol, m-cresol and a creosote mixture for Sauvignon blanc evaluation and additional red berries (fruity) and aluminium sulphate (astringency) for the Merlot evaluation. After that, the panel was presented with the experimental wines and asked to provide attributes which could best describe the wines. Once agreement was reached, the list was reduced to the necessary amount of descriptors, containing enough terms to describe the wine but not too large to confuse the judges. The panel was subsequently trained with a structured 10 cm line scale, demarked with ‘None’ and ‘Intense’ at the extreme left and right sides respectively. This step aimed to get the panellists to understand the intensity scale of each attribute in relation with what they have experienced during training and not as personal knowledge acquired outside the panel environment.

The descriptive study was performed in a single session, set up in different days for Sauvignon blanc and Merlot. Each treatment was presented in 5 replicates. A Latin Square design was used to randomise the distribution of the wines presented to the panellists.

### 4.2.3 Data analysis

Significant attributes and judge outliers were identified by Analysis of Variance (ANOVA) and Tuckerplot using PanelCheck®. Principal Component Analysis (PCA) from Unscrambler® was used to correlate the attributes to the concentration levels of the single volatile compounds.

Panel Performance:

The performance of the assessors was evaluated and outliers eliminated from the judging panel. As demonstrated in Figure 4.1, the assessors showed consensus on 'clean', 'burnt' rubber', 'phenolic' attributes at a 99% confidence level and at 95% for 'astringency' and 'dusty'. The figure represents the 2011 panel performance and similar results were obtained for the 2010 and 2012 performances.

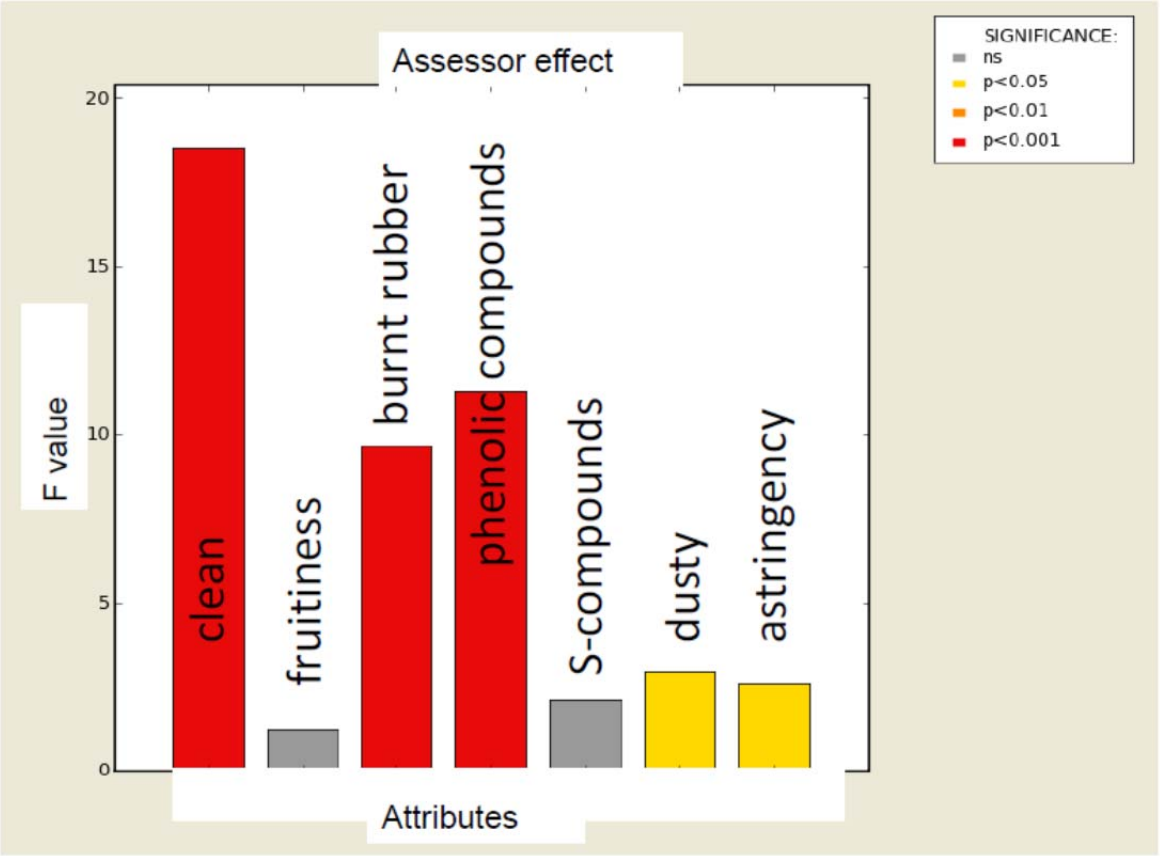



**Figure 4.1.** Assessor effect in the scoring of the 2011 Sauvignon blanc.

To check the panel and the assessor performances in the red wine, Tucker 1 plots were generated. Figure 4.2 illustrates the panel performance in respect to the 'burnt rubber' attribute for the 2010 sensory analyses. The position of the dots on the plot indicates the performance of an individual judge. No noise is present in the system, which would be indicated by the positioning of the assessors close to the middle of the ellipse. All the judges are located on the outer ellipse which indicates 100% explained variance for that specific attribute (Tomic, *et al*., 2010). Similar results were found in 2011 (Figure 4.3) and 2012 for the same attribute. The 'phenolic' attribute was found significant for the 2011 and 2012 Merlot vintages (Figures 4.4 and 4.5), indicating that the panel had a good understanding of the specific character and was able to evaluate the wines according to this descriptor.

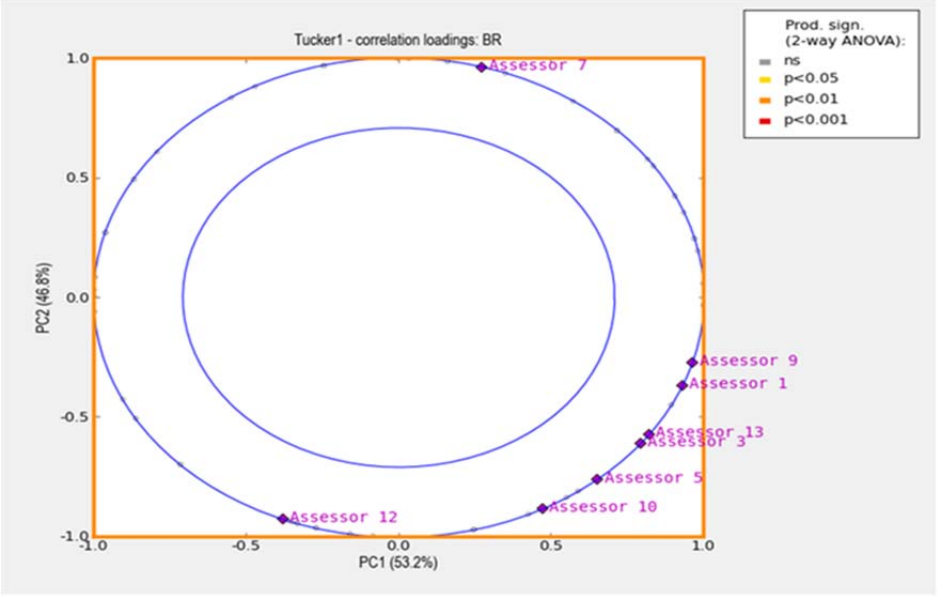



**Figure 4.2.** Tucker1 plot from PanelCheck® illustrating the correlation loadings for the 'burnt rubber' attribute for Merlot 2010 treatments.

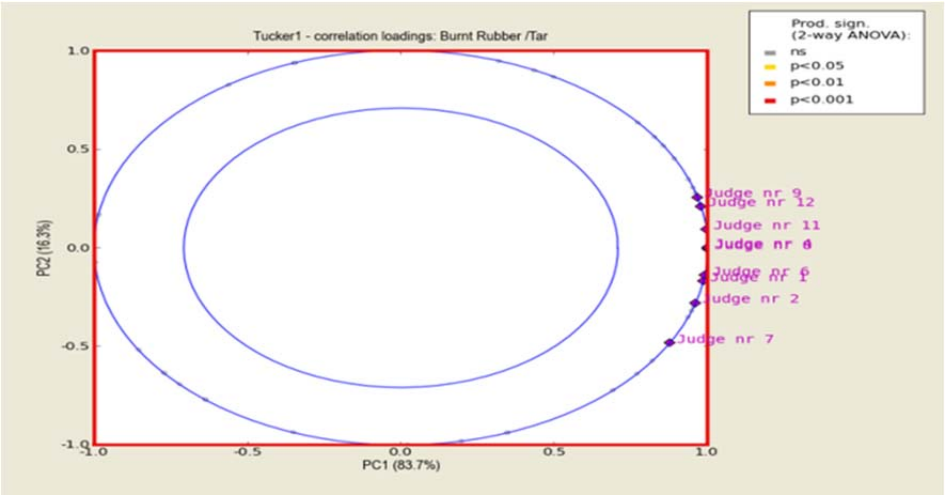



**Figure 4.3.** Tucker1 plot from PanelCheck® illustrating the correlation loadings for the 'burnt rubber/tar' attribute for Merlot 2011

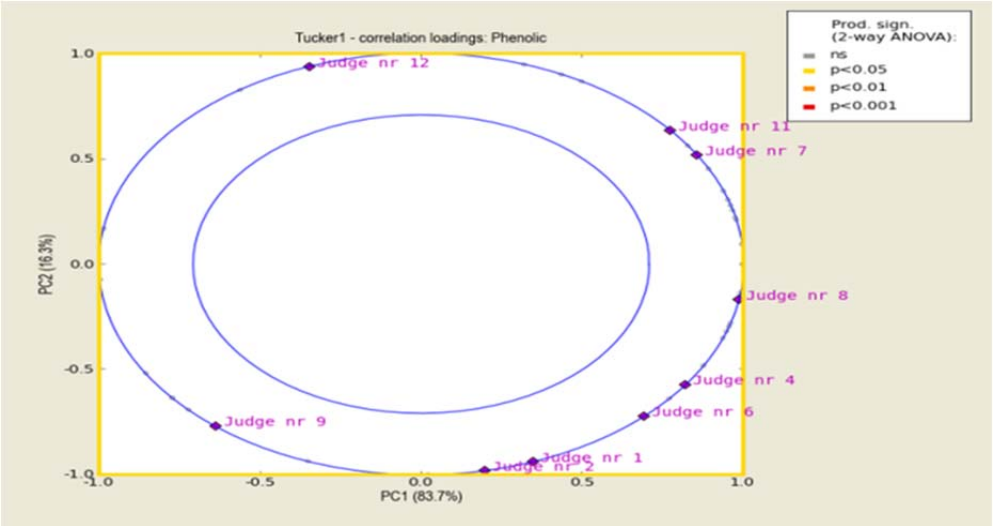



**Figure 4.4.** Tucker1 plot from PanelCheck® illustrating the correlation loadings for the 'phenolic' attribute for Merlot 2011.

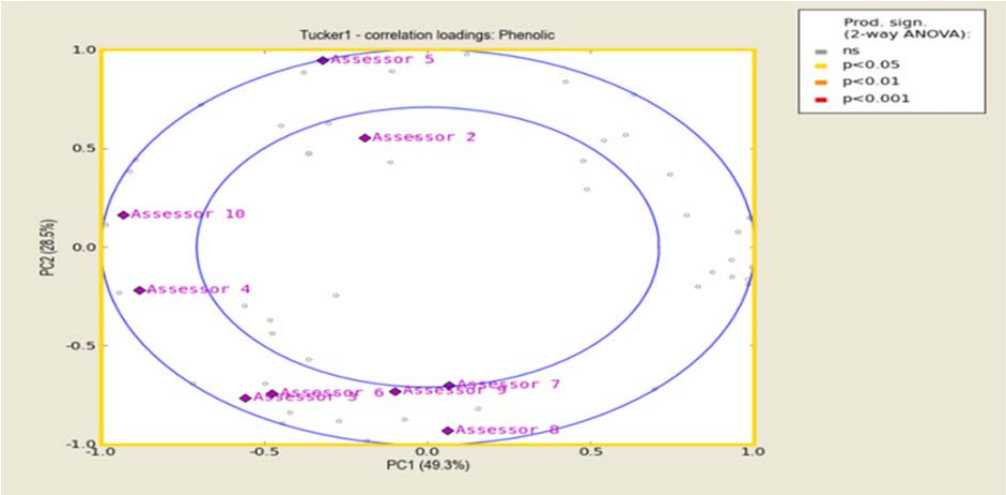



**Figure 4.5.** Tucker1 plot from PanelCheck® illustrating the correlation loadings for the 'phenolic' attribute for Merlot 2012.

## 4.3 RESULTS AND DISCUSSION – CREOSOTE TRIAL

### 4.3.1. Sauvignon blanc

Even with good consistency and repeatability of the panel performance, the Sauvignon blanc treatments showed no effect on the aroma profile of the wine. The spider plot in Fig. 4.6 illustrates the aroma profile of the 2011 Sauvignon blanc characterized mainly by fruitiness. The same was observed for the 2010 and 2012 Sauvignon blanc wines. The results are in line with findings by Kipopoulou, 1999, which stated that the lipophilic nature of PAHs make them likely to be absorbed on the cuticular layer of leaves. This can be applied

to the waxy layer of the grape berries. Assuming that light PAHs and volatile phenols get absorbed on the surface of the fruit, in white wine vinification skin contact is limited and happens only prior to alcoholic fermentation, therefore limiting extraction. For this specific reason the findings were in line with expected outcomes. In the 2010 and 2012 vintage, bitterness in the wine was also profiled; this result could be explained by the higher levels of succinic acid found in those wines. Succinic acid has been described as “less intense (in acidity than tartaric acid), but more complex with bitter and salty notes” (Amerine, Roessler and Ough 1964). It is normally found in wines in concentrations between 0.5 and 1.5g/L, but higher values up to 3.0 g/L have been recorded (De Klerk 2010). In the experimental Sauvignon blanc wines, succinic acid concentrations varied between 0.37 g/L to 0.916 g/L, with no significant differences between treatments. Higher levels were detected for the 2010 and 2012 vintages, above the detection threshold of 0.034 g/L (Thoukis, Ueda and Wright 1964).

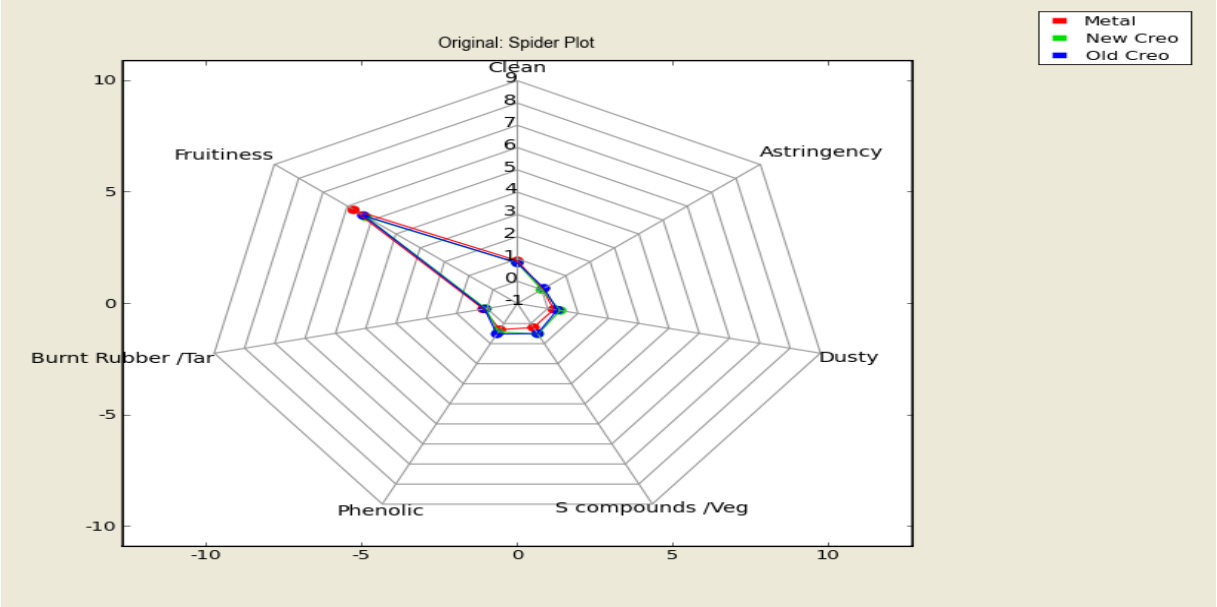



**Figure 4.6.** Spider plot from PanelCheck® illustrating the aroma profile of the Sauvignon blanc 2011 obtained from the 3 different poles treatments.

### 4.3.2. Merlot

Two-way ANOVA was used to assess the importance of the attributes in evaluating differences between treatments. Only attributes that were significant at a minimum of 5% confidence level were chosen, namely ‘burnt rubber’, ‘phenolic’ and ‘chemical’.

A general overview of the effect of treatments on the experimental wines is achieved by applying Principal Component Analysis (PCA) to the data sets. Differences between treatments were significant in the 2010 Merlot wines. New creosoted poles treated wines

were strongly associated with 'burnt rubber', 'creosoted/tar' and 'chemical/Band-aid' attributes, while wines produced with grapes exposed to metal posts were placed the furthest from the malodorous characters (Figure 4.7).

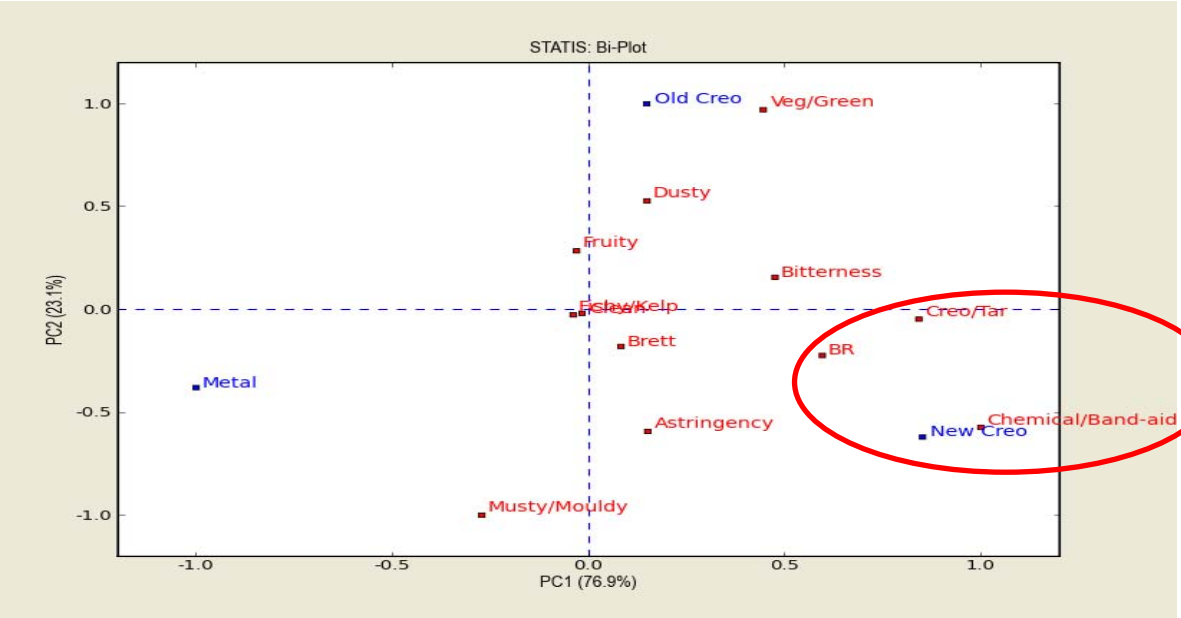



**Figure 4.7** STATIS Bi-plot illustrating the association between treatments and aroma profile of the experimental Merlot 2010.

Similar results were obtained for the 2011 Merlot where the 'burnt rubber/tar' character was once again associated with the new creosoted poles (Figure 4.8). It has to be noted that some of the attributes of interest were merged in the 2011 training session, since the panel struggled to distinguish between 'burnt rubber' and 'tar'. The merging was decided in full agreement with the judges. The impact of the treatments on the wine can also be observed on the STATIS Spider plot (Figure 4.9) where it is associated with the stronger 'burnt rubber' character of the new creosoted poles treatment. The old creosoted poles were once again, as in the 2010 vintage, associated with a 'dusty' character.

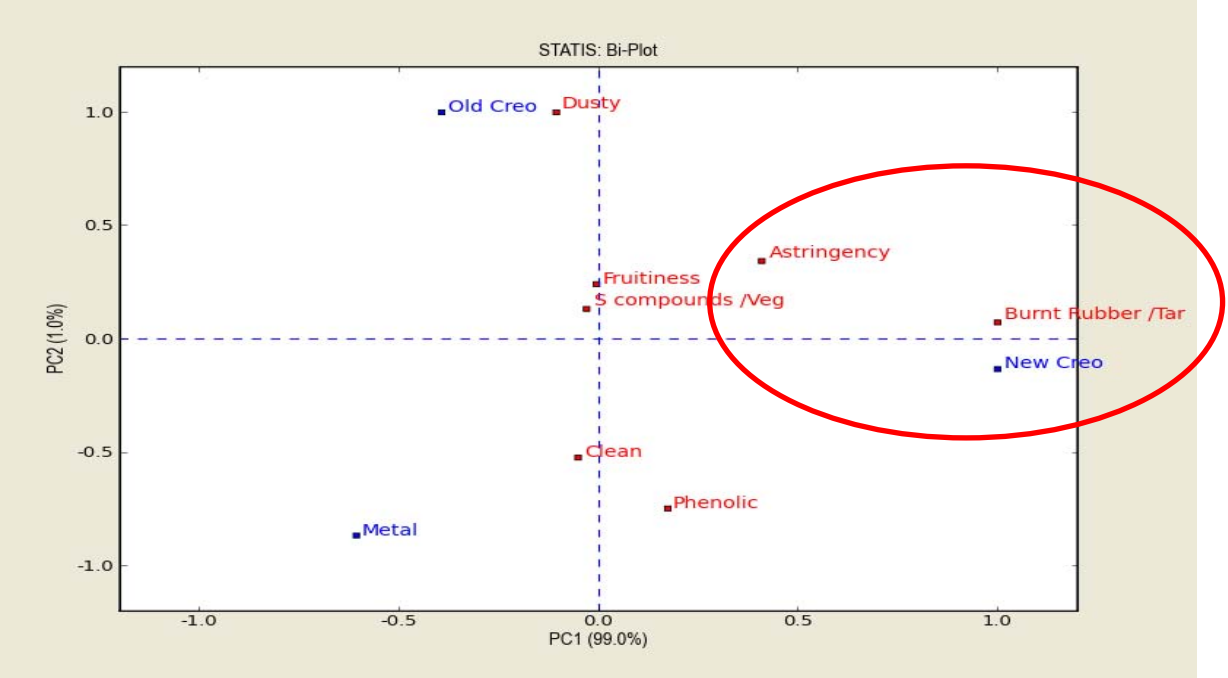



**Figure 4.8.** STATIS Bi-plot illustrating the association between treatments and aroma profile of the experimental Merlot 2011.

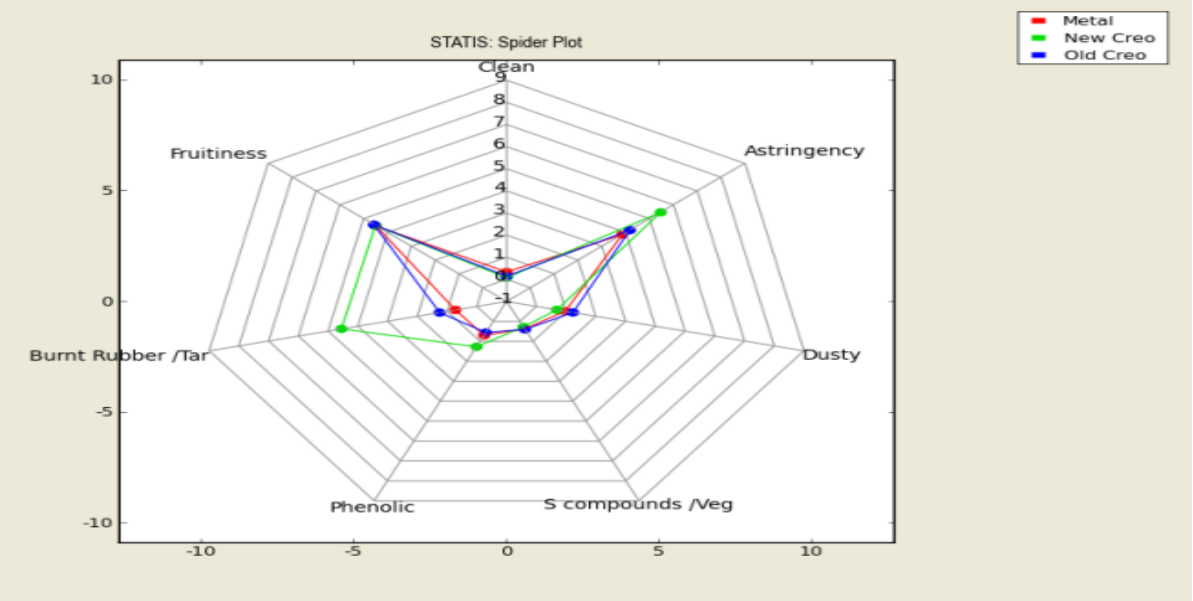



**Figure 4.9.** STATIS Spider plot from PanelCheck® illustrating the aroma profile of the Merlot 2011 obtained from the 3 different poles treatments.

All the above described results were once again confirmed in the 2012 vintage. Analysing the STATIS Bi-Plot (Figure 4.10) it can be noted that new creosoted poles treatment correlates with 'burnt rubber'. A new treatment was introduced in the last growing season which implied the use of high density polyethylene poles. The results are included, but the characteristics of the wine produced with this treatment need further investigation.

From the 2012 sensory evaluation emerged that the HDPE poles (or plastic) are the most associated with the fruitiness of the wine.

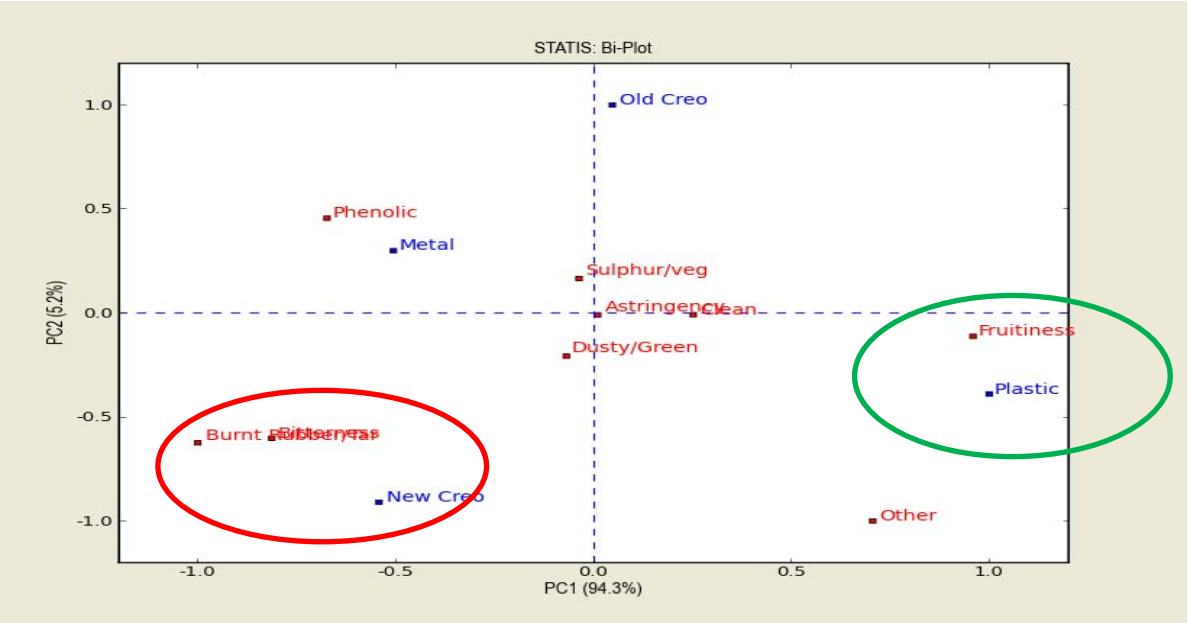



**Figure 4.10.** STATIS Bi-plot illustrating the association between treatments and aroma profile of the experimental Merlot 2012.

## 4.4 CONCLUSIONS – CREOSOTE TRIAL

The effect of vineyard posts on the sensorial characteristics of wine has been investigated in the present study and correlations found between the use of posts and associated wine quality. Creosoted poles were responsible for an off-flavour described as ‘burnt rubber’ and ‘tarry’ in Merlot wines produced with grapes grown in close proximity to the posts and no specific taint was associated with the other posts type used. The ‘burnt rubber’ taint persisted over the three years of the study, despite the inevitable weathering of new poles due to atmospheric conditions, and an expected reduction in their contribution to the taint occurred. Even though creosote products have been banned in Europe and United States of America for any use in food production, South African legislation still allows its use as long as it complies with SABS standards. Following some of the reported findings, new guidelines have been introduced in the Integrated Production of Wine (IPW) certification, which advice against the use of creosoted poles for vineyard trellising. This preliminary, but important guideline is going to bring the South African wine industry a step closer to the most advanced countries in regard to food safety regulations.

## 4.5 SYNERGISTIC STUDY

### 4.5.1 Introduction

It has been well established that volatile phenols at low levels contribute in a positive way to wine complexity but, as has been noted, external contamination can increase the level of those compounds to concentrations which can be detrimental to wine quality (Chatonnet, *et al.*, 1992). Aroma descriptors are very well established for some of the volatile phenols, including guaiacol, the furfurals and 4-ethyl and vinylphenol. Descriptors for 4-ethylphenol (4EP) include 'horsey', 'leather', 'medicinal', 'smokey' (Kennison, *et al*., 2008), while phenol has a strong odour that is 'sickeningly sweet', 'creosote' and 'smoky' (Burdock, 2010). Descriptors are largely unavailable for some of the other phenols we studied, for example, the cresols and the xylenols. A gap in literature has also been identified in the interaction of those compounds with each other, more specifically at levels below detection threshold. Furthermore, no information is available on detection threshold of phenol and 3,4-xylenol in red and synthetic wine.

The present study aims to examine the synergistic effect of phenol, o-cresol, 4-ethylphenol and 3,4-xylenol at 4 different concentrations, as well as establishing odour thresholds for phenol and 3,4-xylenol in synthetic and red wine. This aspect of the study was conducted in order to investigate whether a combination of these phenols, known to be components of the volatile fraction of creosote, was responsible for the 'burnt rubber' taint in South African wines.

### 4.5.2. Materials and methods

#### 4.5.2.1 Standards

Ethanol was purchased from Illovo (Cape Town, SA), sodium hydroxide from Saarchem (Wadeville, SA) and 3,4-xylenol from Riedel-de Haën (Hanover, GE). Tartaric acid, o-cresol and phenol were obtained from Sigma-Aldrich (St. Luis, MO).

#### 4.5.2.2 Base Wine

The red wine used as control was unwooded, single-variety (*cv*. Pinotage) produced by a commercial cellar located in the Western Cape (South Africa) in 2012. Phenol, o-cresol, 4-ethyl phenol and 3,4-xylenol were quantified by a phenol method developed by the Central Analytical Facility of Stellenbosch University in collaboration with the Department of Oenology of the same University and described in chapter 3, section 3.2.3.

The wine was chemically analysed and found clear of any phenol of interest. Other wine chemical parameters were measured by Fourier transform infrared spectroscopy, using a GrapeScan 2000 (FOSS Electronic, Denmark). The results were as follows: 14.92 %v/v alcohol, 5.35 g/L TA, 3.5 pH, 2.48 g/L of residual sugar and 0.46 g/L volatile acidity. The sulphur content was monitored regularly and free $SO_2$ kept at 35 parts per million throughout the experiment.

The synthetic wine solution was prepared using distilled water. Further additions were: 96% ethanol food grade, to bring the solution to 12% alcohol content; 4.5 g/L tartaric acid and sodium hydroxide to bring the pH value to 3.5.

A standard stock solution of each volatile phenol was prepared fresh daily in synthetic wine to a concentration of 1000 parts per million (µg/L) and used for spiking of the red and synthetic wine during the experiment. Table 4.2 illustrates the concentrations used for the interaction study.

**Table 4.2.** Concentrations used for the volatiles compounds in the interaction study (values in µg/L).

| Level | Phenol | o-Cresol | 3,4-Xylenol | 4-Ethyl Phenol |
|---|---|---|---|---|
| **0** | 0 | 0 | 0 | 0 |
| **1** | 60 | 20 | 70 | 55 |
| **2** | 130 | **30**[a] | **640**[c] | 260 |
| **3** | **7100**[a] | 50 | 800 | **600**[b] |

(a) Odour DT in aqueous solution at 10% ethanol
(b) DT in red wine
(c) Determined prior to this study – method based on ASTM E-679

### 4.5.2.3 Experimental design

A panel of 9 judges was selected for the synergistic studies: 2 males and 7 females all with moderate to good experience in wine evaluation and working for the Department of Oenology of the Stellenbosch University, either as academics or postgraduate students. Descriptive analysis was employed to investigate masking and synergistic interactions. The panel of judges began its training by generating the descriptors which best described the wine spiked with the individual compounds and as a mixture of the four phenols. In the following training sessions, standards reflecting the attributes generated were prepared to facilitate the training and the list of attributes reduced to avoid confusion. The final step aimed to get the panellists to understand the intensity scale of each attribute in relation with

what they have experienced during training and not as personal knowledge acquired outside the panel environment. An unstructured line scale was used to quantify the attributes with regard to their intensity; this was done to avoid bias in the panellists' judgement.

The descriptive study was performed in 3 different sessions. During the first one, the judges evaluated the wines spiked with individual compounds at 3 different levels. Each sample was presented in triplicates. A modified Central Composite Design was used for the following two sessions, in order to evaluate the interaction of the compounds with each other in all possible combinations of concentrations. In the second and third descriptive sessions the 9 wine samples were also presented in triplicate. A control sample was supplied to each judge during the descriptive evaluation. The intensity of the attributes was evaluated on a 100 mm unstructured scale, demarked with 'None' and 'Intense' at the extreme left and right sides respectively.

#### 4.5.2.4 Data analysis

Significant attributes and judge outliers were identified by Analysis of Variance (ANOVA) and Tuckerplot using PanelCheck®. Principal Component Analysis (PCA) from Unscrambler® was used to correlate the attributes to the concentration levels of the single phenols as well as the correlations between them. Furthermore, Surface Response was employed as part of the Central Composite Design strategy using Statistica® software.

### 4.5.3 Results and discussion

Descriptors of the compounds in wine were established during the training sessions and included 'tar-like' and 'chemical' for the cresols, to 'sick sweet' and 'medicinal' for the xylenols (Table 3). Descriptive analysis was also used to determine interaction between phenol, o-cresol, 3,4-xylenol and 4-ethylphenol at four different concentrations according to a modified Central Composite Design in order to include all possible combinations. The spiked wines were characterized by very different attributes when evaluated on a single compound (see Table 4.3) and not all the phenols contributed negatively to the profile of the wine.

**Table 4.3.** Sensory attributes of spiked red wine generated by judges during training.

| Compound | Descriptor |
|---|---|
| **Phenol** | Artificial sweet, berry jam, floral |
| **o-cresol** | Medicinal, smoky, moth-balls |
| **3,4-xylenol** | Sick sweet, artificial sweet, medicinal, |
| **4-ethyl phenol** | Horsey, medicinal, band-aid, leather |
| **4 phenols mix at detection threshold** | Medicinal, horsey, burnt rubber, smoky, nail polish remover |

ANOVA produced five statistically significant attributes for the interaction of the four compounds, namely 'medicinal/Band-aid', 'horsey/leathery', 'sick sweet', 'burnt rubber' and 'moth-balls'. Furthermore, the 'smoky' descriptor was also significant but only in regard to o-cresol, when used on its own. Phenol in red wine was described as 'floral and sweet' (Parker et al., 2010) In the present study similar results were observed: spiking of pinotage, with phenol only, enhanced the berry jam character of the wine, placing the wine samples at the bottom, right quadrant of the Bi-plot (Figure 4.11), away from the faulty descriptors.

Surface Response Plots (Statistica®) were utilized as part of a Central Composite design in order to better explain the results. Significant interactions were observed between the malodorous phenols. While 4-ethylphenol was found to be the most significant compound with regard to the 'medicinal/band-aid' and 'horsey/leathery' attributes; once it was combined in a mixture with increasing concentrations of phenol (Figure 4.11) and o-cresol (Figure 4.12) the interaction caused the intensity of the taint to increase considerably.

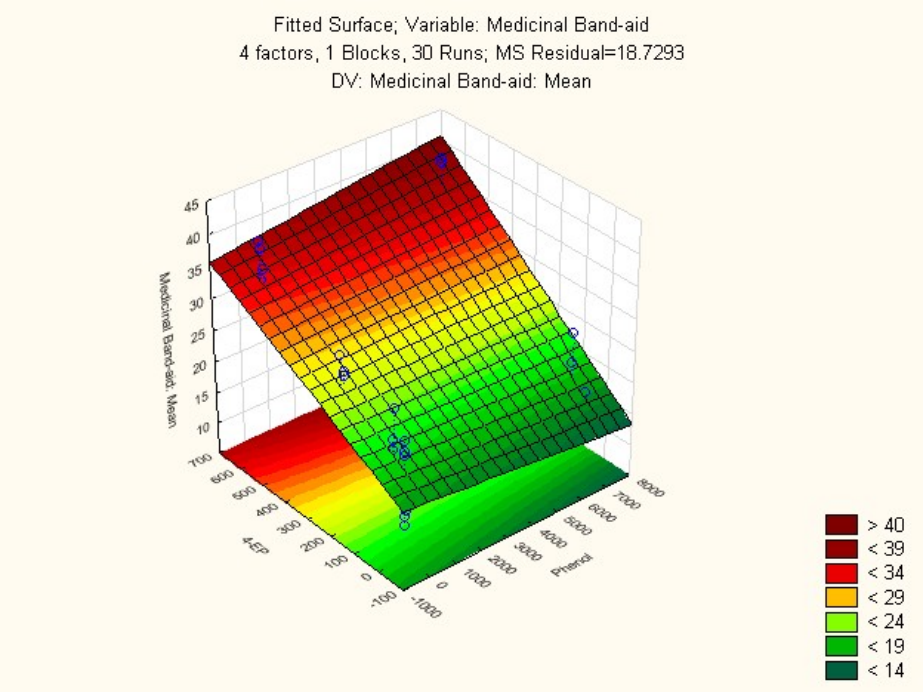



**Figure 4.11.** Surface Response plot illustrating the interaction between 4EP and phenol in regard to the 'Medicinal/Band-aid' attribute.

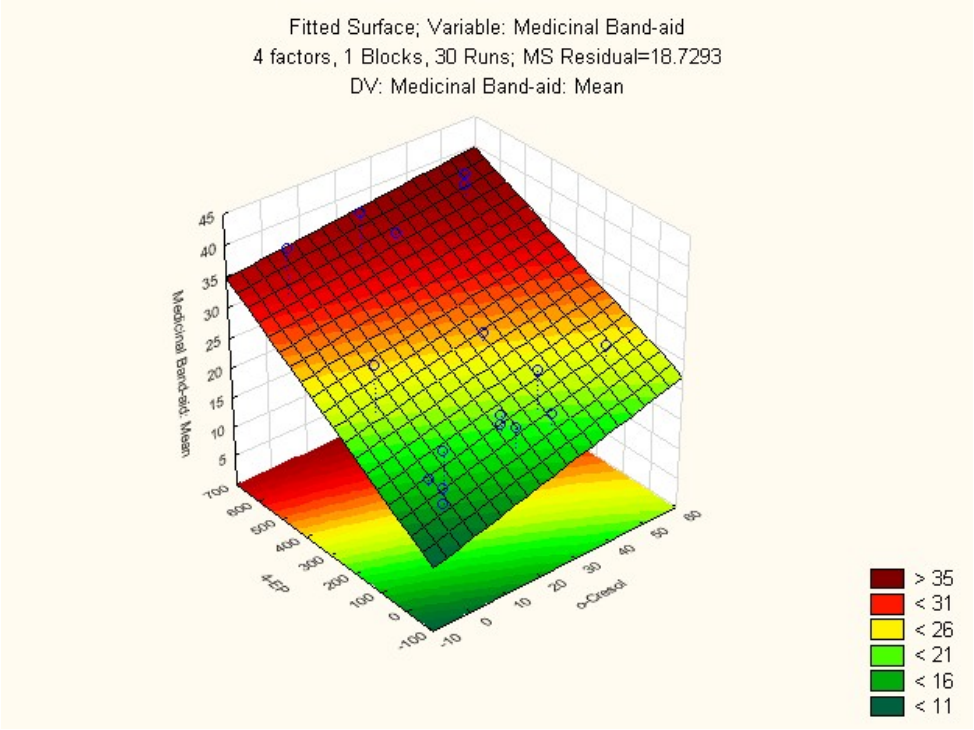



**Figure 4.12.** Surface Response plot illustrating the interaction between 4EP and o-cresol in regard to the 'Medicinal/Band-aid' attribute.

O-cresol was the only compound associated with the 'smoky/ash' attribute and no interaction between o-cresol and 4-ethylphenol (Figure 4.13) or with 3,4-xylenol were observed. However, once o-cresol was combined with phenol at increasing concentrations, an enhancement of this character can be observed, despite the positive contribution ('artificial sweet, berry jam, floral') of phenol as individual compound (Figure 4.14).

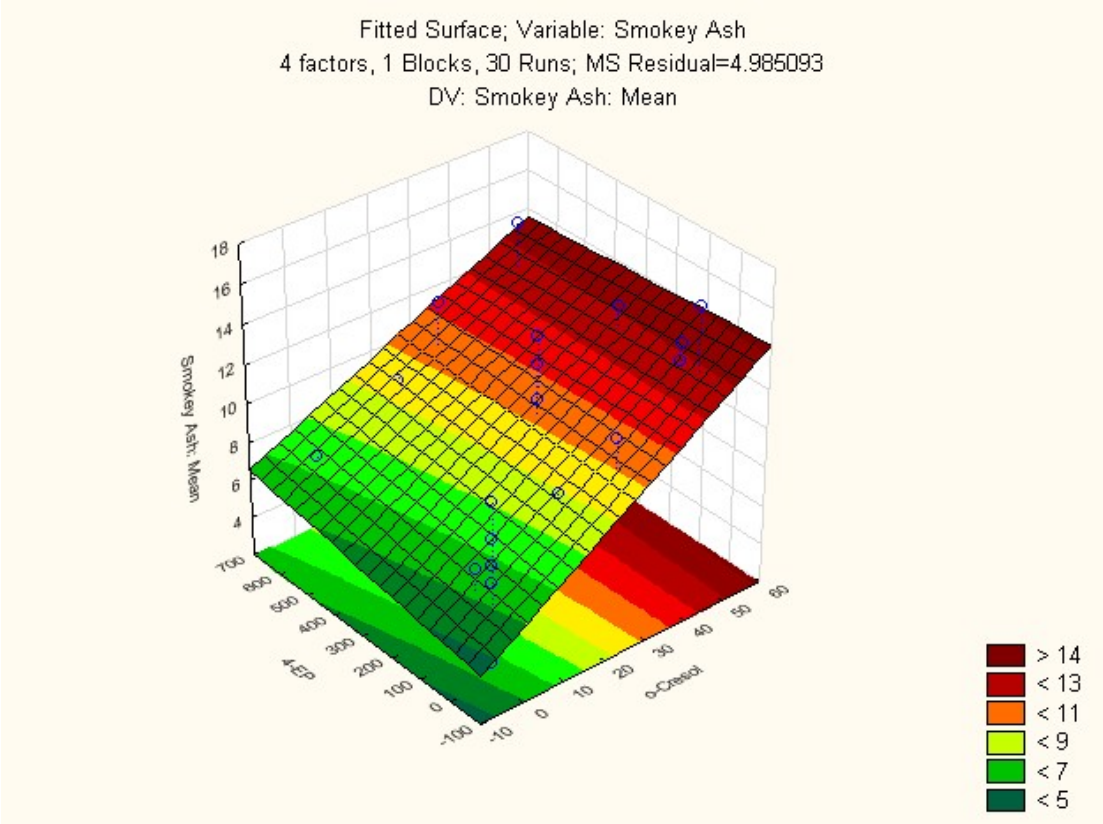



**Figure 4.13.** Surface Response plot illustrating the interaction between 4EP and o-cresol in regard to the 'Smoky/Ash' attribute.

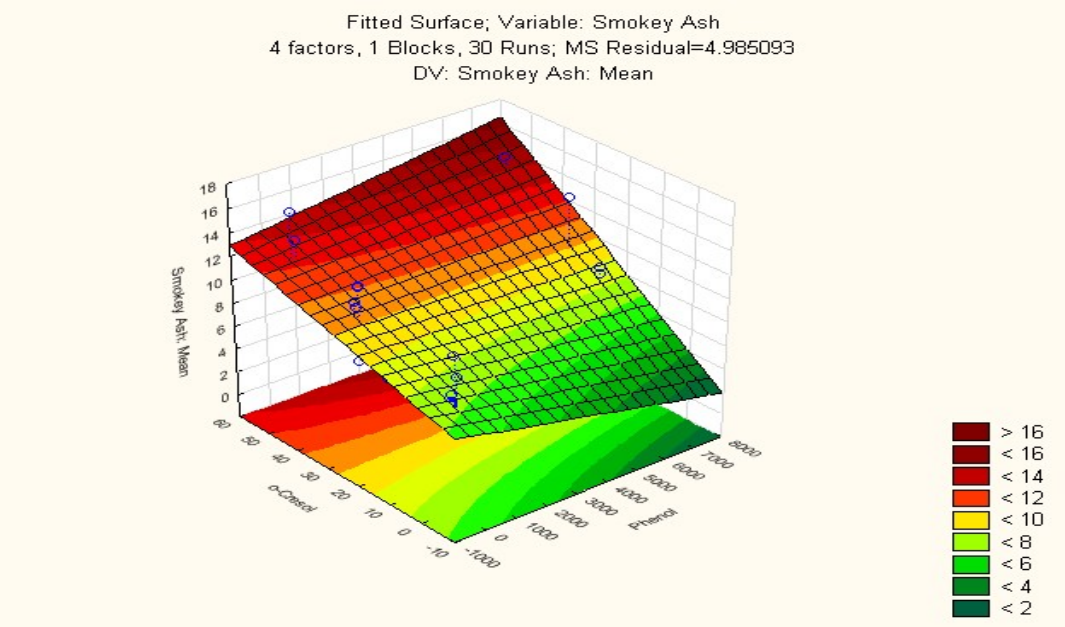



**Figure 4.14.** Surface Response plot illustrating the interaction between o-cresol and phenol in regard to the 'Smoky/ash' attribute.

Figure 4.15 illustrates the Surface Response plot of the interaction between 3,4-xylenol and o-cresol in regard to the 'sick sweet' attribute. O-cresol and 3,4-xylenol were both statistically significant attributes on their own. An interesting phenomenon is observed at increasing concentrations of both: the intensity of the 'sick sweet' character is diminished. This effect can probably be ascribed to the masking effect of the smoky character of o-cresol on 3,4-xylenol.

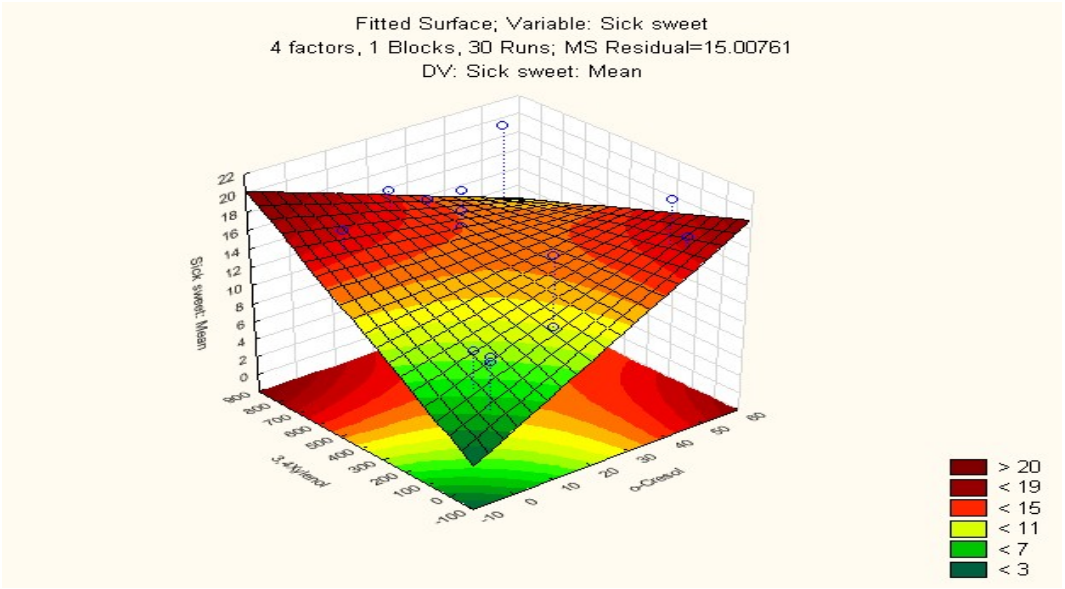



**Figure 4.15.** Surface Response plot illustrating the interaction between 3,4 xylenol and o-cresol in regard to the 'Sick sweet' attribute.

It has to be noted that all the descriptors were generated by the panel during training and most of the attributes fitted the spiked wine when present as individual as well as mixed compounds. Only one attribute emerged once the compounds were mixed together as a secondary taint. The 'burnt rubber' descriptor appeared to be an ideal way to describe the character of the wines spiked with the mixture of phenols. 4-ethylphenol was found to play a major role in this regard: the interaction between 4-ethylphenol and phenol was statistically significant (Figure 4.16) and a similar trend was observed for the combination of 4-ethyl-phenol with o-cresol (Figure 4.17).

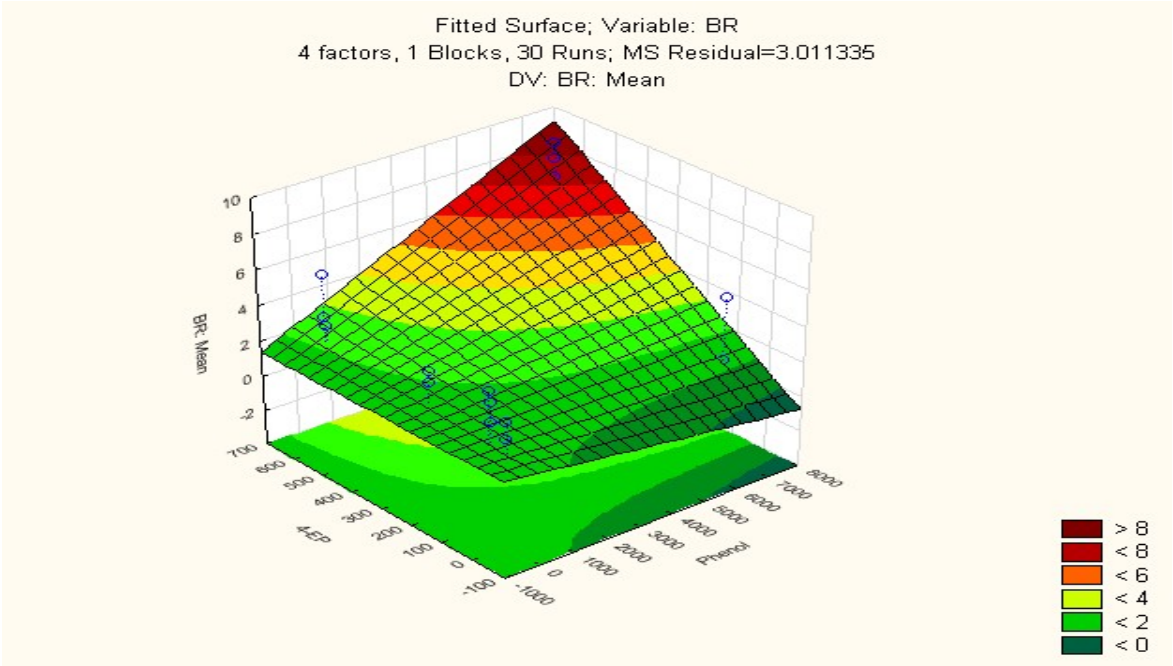



**Figure 4.16.** Surface Response plot illustrating the interaction between 4EP and phenol in regard to the 'Burnt Rubber' (BR) attribute..

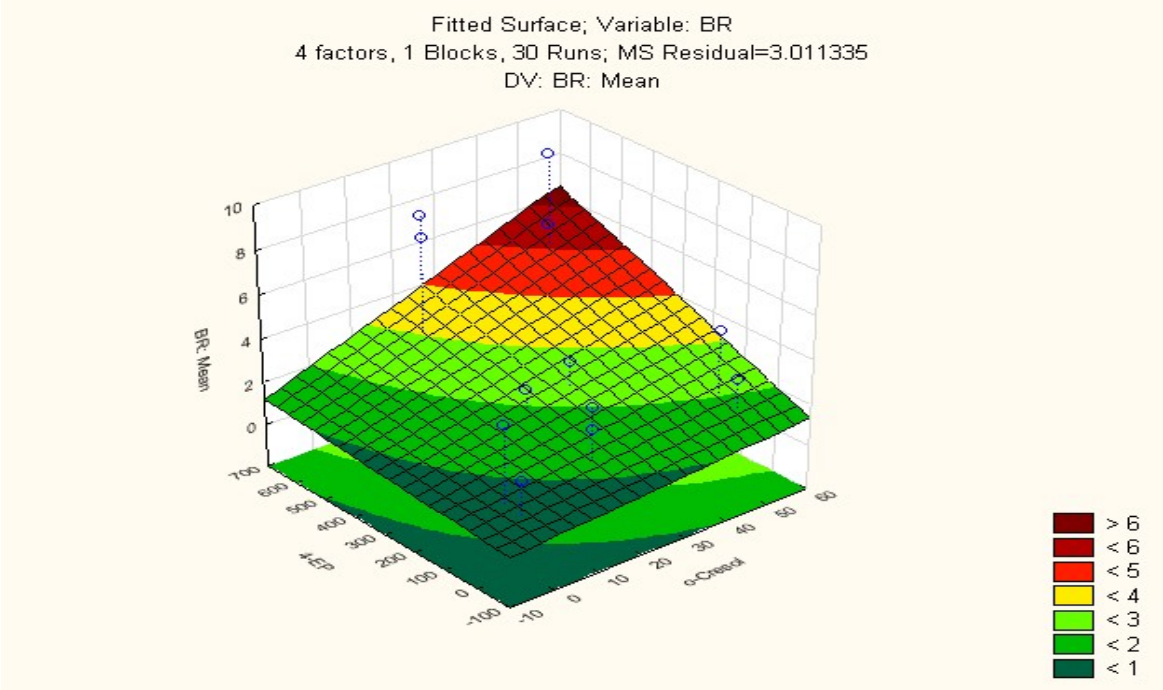



**Figure 4.17.** Surface Response plot illustrating the interaction between 4EP and o-cresol in regard to the 'Burnt Rubber' (BR) attribute.

To summarize the interaction between all the compounds, which cannot be represented in the Surface Response plots, the Bi-plot generated with Unscrambler® was used. Each wine, with its combination of compounds, is represented by a four digit code. The compounds are in the following order: phenol, o-cresol, 3,4-xylenol and 4-ethylphenol. The concentrations are from 0 (not present) to 3 (highest level) as described in Table 4.2. The spiked wine samples appeared to divide themselves along PC1, probably showing that the attributes were responsible for this variation in the data set. PC2 appeared to correlate to spiking concentrations, spreading the samples diagonally from a bottom/left to a top/right direction. It has to be noted that PC1 and PC2 combined, explained 72% of the variance in the system, indicating that attributes and concentrations are the most important elements plying a part in the characterization of the wines.

'Smoky/ash' and 'moth-ball' were significant attributes for o-cresol when spiked on its own, but higher intensity in the 'medicinal/band-aid' character was also observed at increasing concentrations of o-cresol.

'Medicinal' and 'horsey/leathery' were the most significant attributes for 4-ethylphenol as single compound, but these attributes resulted to be the dominant ones also as the concentrations of the other three compounds were increased, as interaction between all of them.

A secondary attribute emerged once the compounds were mixed together. A ‘burnt rubber’ descriptor, never present as attribute for any of the pinotage samples spiked with individual phenols, appeared to describe the character of the wines spiked with the mixture of phenols very well.

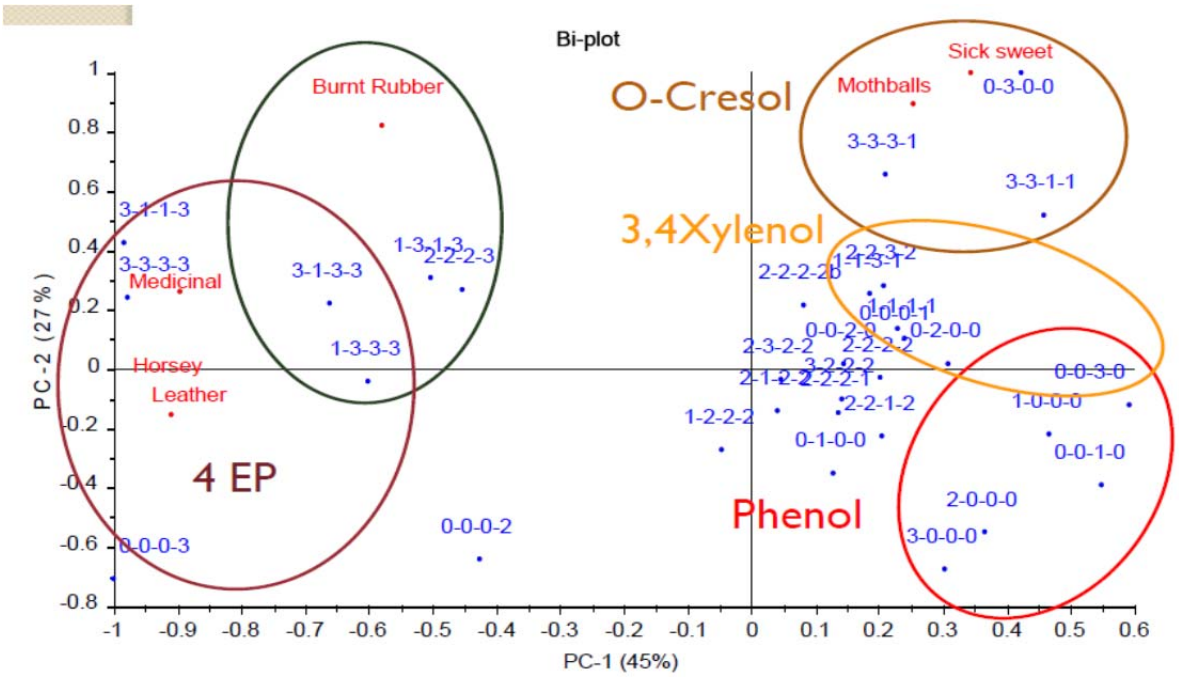



**Figure 4.18.** Principal component Bi-plot of descriptive attributes in respect to the concentration levels of the spiked red wine. The four digit code represents the levels of spiking of each compound in the following order: phenol, o-cresol, 3,4-xylenol and 4-ethylphenol. The concentrations are from 0 (not present) to 3 (highest level) as described in Table 4.2.

### 4.5.4 Conclusions

The sensory attributes of 4-ethylphenol, o-cresol, 3,4-xylenol and phenol were confirmed in the study to be as reported in the literature, but secondary taints have been found as a result of their interaction. In particular, 4-ethylphenol appeared to be a key contributor to the ‘burnt rubber’ odour, especially in combination with phenol and o-cresol. Furthermore, a synergistic effect was discovered between o-cresol and 3,4-xylenol with regard to the ‘sick sweet’ character. Decreasing intensity of the attribute at increasing concentration of both compounds could be ascribed to the masking effect of the strong ‘smoky’ character of o-cresol over the milder ‘sick sweet’ attribute.

During the training process, some difficulties were encountered over descriptors like ‘nail-polish remover’ and ‘glue’. These attributes were not included in the list used for the

scoring of the wine, because agreement was not reached on their relevance for the project. Base wine used for the present study was a pinotage, which had fairly strong odour characteristics. For future studies we would suggest that a more neutral wine should be used in order to minimize confusion amongst the panel. Further studies should investigate the validity of these findings in respect to other cultivars.

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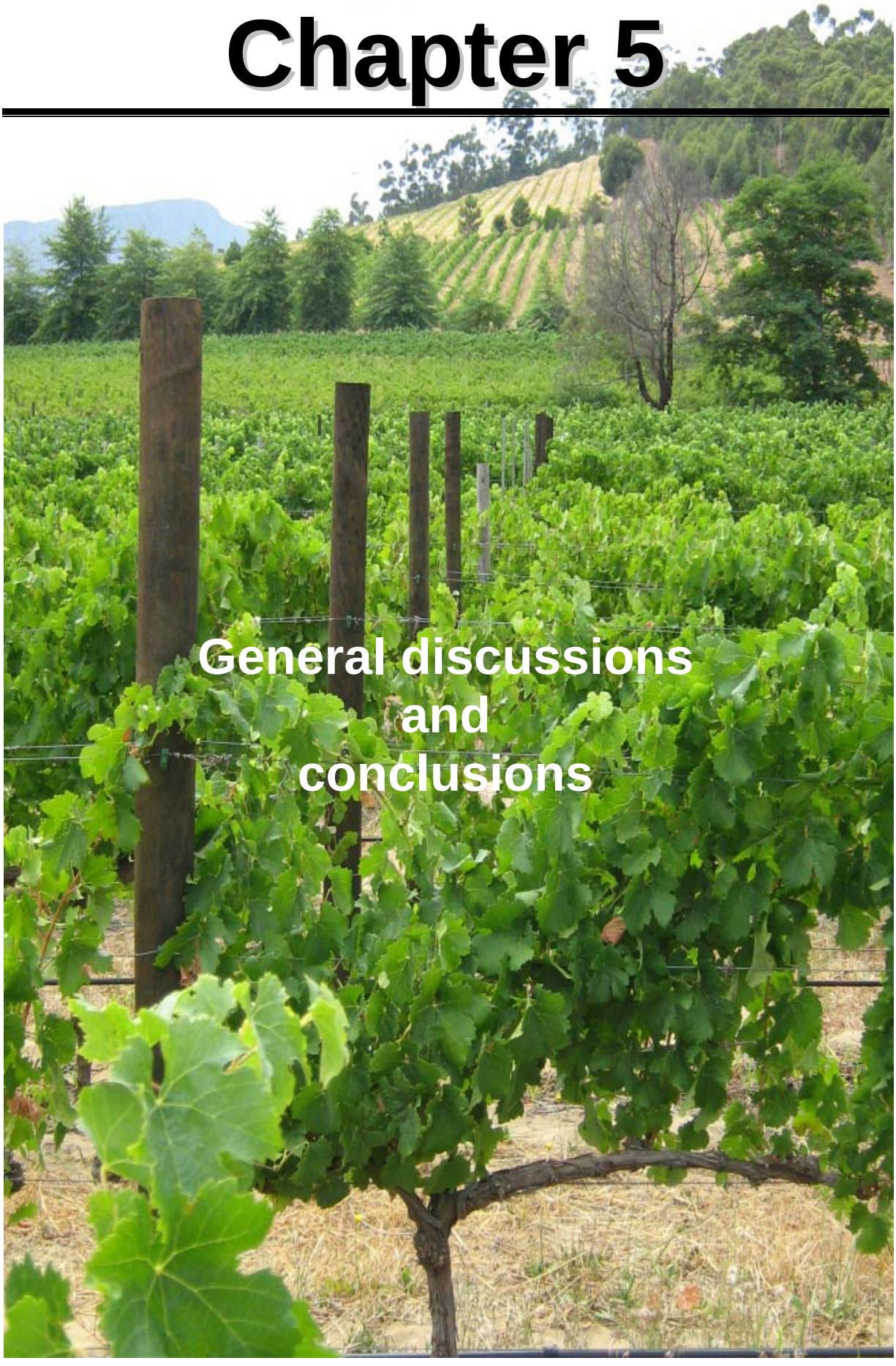
# Chapter 5



## General discussions and conclusions

# 5. GENERAL DISCUSSION AND CONCLUSIONS

## 5.1 CONCLUSIONS AND FUTURE PROSPECTS

Trellis systems, or training systems, are an integral part of the agricultural infrastructure, used for centuries to increase the yield and quality of various crops. A wide range of materials are used in viticulture for the production of supporting posts. Coal tar creosote is a very common wood preservative used in South Africa, due to the high efficiency in protecting utility poles against climatic and biological degradation. The composition of the creosote mixture is very complex and can differ depending on the origin of the coal and the method used for distillation (Melber, *et al.,* 2004). Some of the compounds in the mixture display strong odour characteristics (Choudhary, *et al.,* 2002) and it is for this specific reason that creosote became the focus of attention for the present study.

The overall aim of this study was to determine if there is a relationship between creosoted posts usage and 'burnt rubber' taint in wine. Accumulation of phenols, cresols, xylenols and PAHs on the grapes could lead to the extraction of the unwanted compounds into the wine during alcoholic fermentation, creating quality and sensory problems. As outlined in Chapter 2, it is clear that extensive literature deals with methods of analysis of PAHs and malodorous phenols using both Gas Chromatography, as well as High Performance Liquid Chromatography. Subjects of scrutiny are mainly some categories of foodstuff linked to environmental issues. Investigations included soil, water, seafood and some vegetables, in particular olives derivatives such as olive oil. Due the increasing number of strict regulations dealing with food safety, more and more foodstuff categories have been placed under scrutiny. An effective and efficient sample preparation method for wine and a HPLC-DAD method was developed as part of this project, with the aim of equipping the wine industry with a screening method for identifying specific organic contaminants. Volatile phenols, on the other hand, despite not representing a food safety issue, can represent a threat to wine quality if present at high concentrations, as they will dominate and impart smoky, charred or burnt aromas that detract from the quality of the wine (Chatonnet *et al*., 1992). An additional advantage of this project was a GC-MS method developed to investigate the concentration of volatile phenols in tainted wine.

In Chapter 3, it was demonstrated that Sauvignon blanc experimental wines contained only one of the PAHs of interest, namely chrysene, at levels ranging from 3.8 to 4.7 µg/L (Table 7). The chrysene that was present in all the Sauvignon blanc samples may have been absorbed via the roots rather than through the aerial parts of the plant, but this aspect bears further investigation. Fismes *et al.* (2002) identified root uptake as the main pathway for high molecular PAHs for vegetables, grown on an industrial contaminated soil (Fismes *et al.,*

2002). According to the Scientific Committee on Food, “BaP can be used as a marker for the occurrence and effect of carcinogenic PAHs in food including... chrysene” (Scientific Committee on Food 2002). The maximum levels allowed ranges between 5.0 µg/kg of wet weight for smoked meats to 10.0 µg/kg for bivalve molluscs (The Commission of the European Communities 2006). In light of such regulations, the concentrations of chrysene found in the experimental wines falls within the prescribed parameters for certain food products. Since no other PAH compounds were found in the samples analysed, it can be concluded that the experimental wines were safe for human consumption and complied with EU regulations. Merlot experimental wines did not contain any PAH: if extraction of PAHs occurred, like in the case of Sauvignon blanc, precipitation of the compounds of interest could have happened during lees contact. However these results are not conclusive.

The effect of vineyard posts on the sensorial characteristics of wine has been discussed in Chapter 4 and a direct link was found: creosoted poles were responsible for an off-flavour described as ‘burnt rubber’ and ‘tarry’ in Merlot wines produced with grapes grown in close proximity to the posts. The taint persisted from year to year, despite the inevitable weathering of the poles due to atmospheric conditions. Volatile phenols were detected in both Sauvignon blanc and Merlot experimental wines at concentrations below detection threshold. Nevertheless, the wines produced by grapes grown next to the new creosote poles, displayed a ‘burnt rubber’, ‘phenolic’, ‘smoky’ and ‘medicinal’ characteristics when subject to sensory analysis. A parallel trial was conducted to investigate possible synergistic effect of some of those phenols which confirmed that even at concentrations below their individual detection thresholds, volatile phenols may ‘work together’ to create taints like ‘burnt rubber’. Furthermore, the characters of 4-ethylphenol, o-cresol, 3,4-xylenol and phenol were confirmed as per reported literature, but a secondary taint was found as a result of their interaction. In particular, 4-ethylphenol appeared to be a key contributor to the ‘burnt rubber’ odour, especially in combination with phenol and o-cresol. Furthermore, a synergistic effect was discovered between o-cresol and 3,4-xylenol with regard to the ‘sick sweet’ character. However, these results are preliminary and further studies should investigate the validity of these findings in respect to other cultivars.

Recommendations for future investigations should also include: an evaluation of cumulative effect of creosoted posts in a fully trellised vineyard; a study on the effects of alternative wood treatments (such as CCA/Tanlith E) on wine in terms of quality and chemical composition; analyse the composition of wine made from grapes exposed to creosote using an untargeted and/ or GCO approach in order to establish if other compounds are significant in the burnt rubber/tarry aroma, and to repeat sensory studies with a more neutral wine and alternative compounds.

Even though creosote products have been banned in Europe and United States of America for any use linked to food production, South African legislation still allows its use as long as it complies with SABS standards. Following some of the reported findings, new guidelines have been introduced in the Integrated Production of Wine (IPW) certification, which advise against the use of creosoted poles for vineyard trellising. This preliminary but important guideline will bring the South African wine industry a step closer to the fulfilling the obligations for food safety as required by the legislation of our major export partners.

## 5.2 LITERATURE CITED

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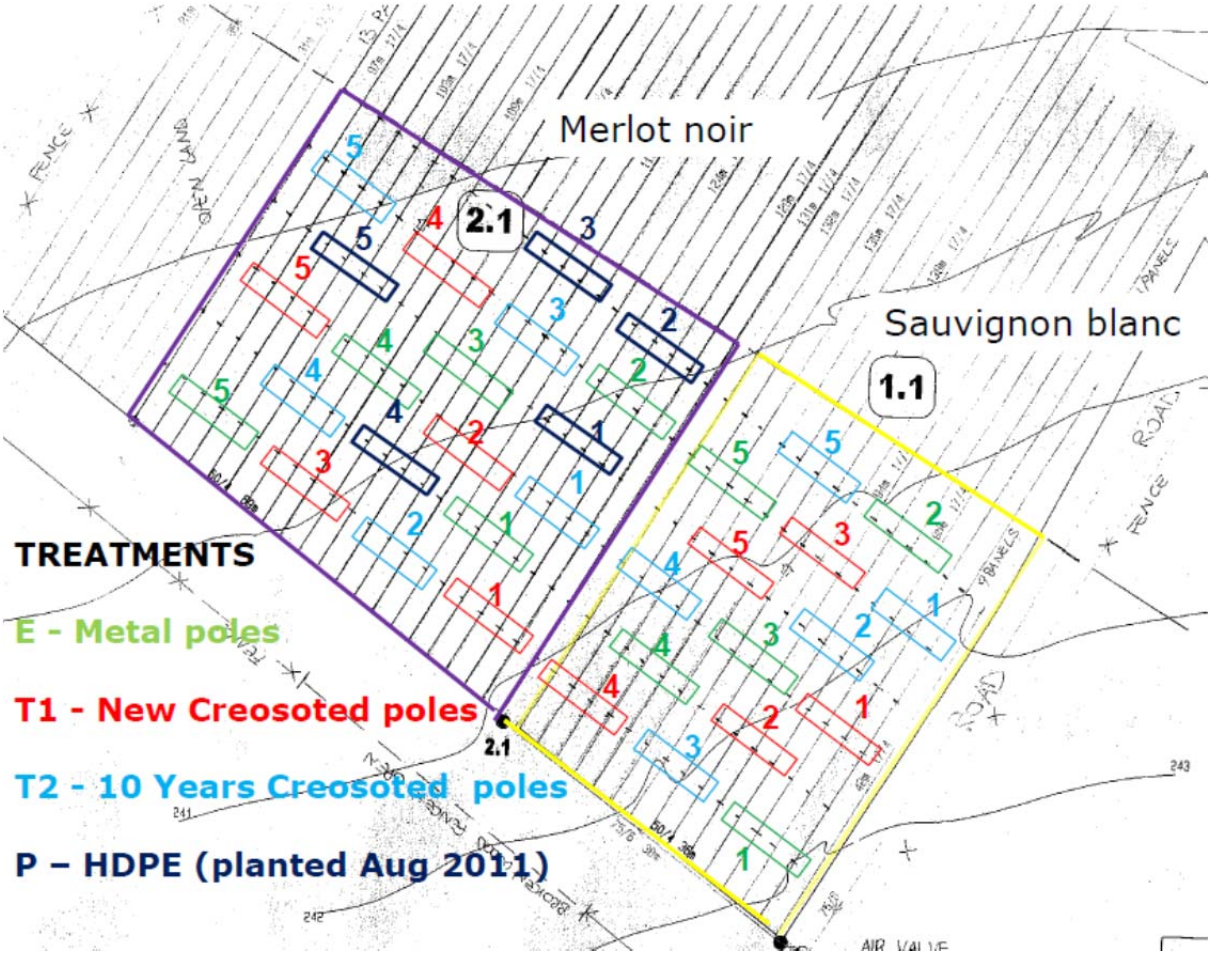
# 6. APPENDICES

**APPENDIX A (**Experimental vineyard layout)



## **APPENDIX B:** Experimental wines GrapeScan 2000 (FOSS) results

| Wine | pH | VA | TA | Malic A | Glucose | Fructose | Ethanol |
|---|---|---|---|---|---|---|---|
| | | g/L | g/L | g/L | g/L | g/L | v/v |
| SB10 E1 | 3.47 | 0.37 | 4.85 | 1.95 | 0.08 | 2.45 | 13.3 |
| SB10 E2 | 3.35 | 0.37 | 5.1 | 1.88 | 0.13 | 2.78 | 13.54 |
| SB10 E3 | 3.36 | 0.36 | 5.12 | 1.92 | 0.24 | 2.42 | 13.68 |
| SB10 E4 | 3.36 | 0.34 | 5.26 | 2.31 | 0.19 | 1.46 | 13.16 |
| SB10 E5 | 3.28 | 0.36 | 5.42 | 2.18 | 0.06 | 1.87 | 12.77 |
| SB10 T21 | 3.4 | 0.43 | 5.15 | 2.06 | 0.23 | 2.01 | 13.63 |
| SB10 T22 | 3.37 | 0.41 | 5.11 | 2.06 | 0.24 | 4.31 | 13.69 |
| SB10 T23 | 3.43 | 0.43 | 5.06 | 2.14 | 0.28 | 1.51 | 13.88 |
| SB10 T24 | 3.34 | 0.37 | 5.11 | 1.97 | -0.26 | 1.4 | 13.12 |
| SB10 T25 | 3.35 | 0.35 | 5.22 | 2.29 | 0.04 | 2.05 | 12.86 |
| SB10 T11 | 3.44 | 0.47 | 4.8 | 1.83 | 0.31 | 2.26 | 13.91 |
| SB10 T12 | 3.44 | 0.43 | 4.95 | 1.82 | 0.42 | 1.59 | 14.1 |
| SB10 T13 | 3.36 | 0.38 | 5 | 1.98 | 0.06 | 1.88 | 13.61 |
| SB10 T14 | 3.35 | 0.36 | 5.53 | 2.75 | -0.09 | 2.37 | 12.93 |
| SB10 T15 | 3.37 | 0.36 | 5.19 | 2.15 | 0.29 | 1.8 | 13.34 |
| SB11 E1 | 3.17 | 0.34 | 5.86 | 1.51 | -0.02 | 1.6 | 12.94 |
| SB11 E2 | 3.01 | 0.35 | 6.17 | 1.33 | 0.06 | 2.68 | 14 |
| SB11 E3 | 3.04 | 0.39 | 6.03 | 1.54 | 0.16 | 4.56 | 14.04 |
| SB11 E4 | 3.05 | 0.35 | 6.11 | 1.84 | 0.21 | 3.54 | 13.92 |
| SB11 E5 | 2.97 | 0.38 | 6.3 | 1.59 | 0.39 | 3.26 | 13.82 |
| SB11 T21 | 3.12 | 0.33 | 5.74 | 1.61 | 0.29 | 2.65 | 13.38 |
| SB11 T22 | 3.11 | 0.36 | 5.7 | 1.6 | 0.65 | 4.76 | 13.93 |
| SB11 T23 | 3.17 | 0.36 | 5.56 | 1.55 | 0.33 | 2.91 | 13.41 |
| SB11 T24 | 3.07 | 0.34 | 5.82 | 1.53 | 0.02 | 2.45 | 13.42 |
| SB11 T25 | 3.15 | 0.33 | 5.61 | 1.54 | 0.3 | 3.43 | 13.6 |
| SB11 T11 | 3.12 | 0.35 | 5.75 | 1.41 | 0.36 | 3.61 | 14.13 |
| SB11 T12 | 3.1 | 0.34 | 5.73 | 1.38 | 0.44 | 4.09 | 14.15 |
| SB11 T13 | 3.04 | 0.32 | 5.93 | 1.59 | 0.46 | 6.05 | 13.78 |
| SB11 T14 | 3.12 | 0.32 | 5.86 | 1.63 | -0.05 | 2.52 | 13.28 |
| SB11 T15 | 3.07 | 0.34 | 5.87 | 1.72 | 0.25 | 5.53 | 13.72 |
| SB12 E1 | 3.39 | 0.29 | 5.15 | 1.67 | 0.27 | 2.28 | 14.01 |
| SB12 E2 | 3.27 | 0.33 | 5.24 | 1.55 | 0.89 | 4.49 | 14.87 |
| SB12 E3 | 3.23 | 0.31 | 5.43 | 1.75 | 0.53 | 4.16 | 14.36 |
| SB12 E4 | 3.23 | 0.31 | 5.64 | 2.01 | 0.46 | 3.31 | 14.05 |
| SB12 E5 | 3.22 | 0.31 | 5.45 | 1.85 | 0.48 | 4.58 | 14 |
| SB12 T21 | 3.28 | 0.33 | 5.37 | 1.74 | 0.54 | 3.59 | 14.28 |
| SB12 T22 | 3.26 | 0.33 | 5.19 | 1.58 | 0.76 | 5.16 | 14.28 |
| SB12 T23 | 3.3 | 0.31 | 5.26 | 1.77 | 0.49 | 4.14 | 14.37 |
| SB12 T24 | 3.19 | 0.29 | 5.58 | 2.05 | 0.46 | 3.96 | 13.82 |
| SB12 T25 | 3.26 | 0.31 | 5.18 | 1.76 | 0.75 | 4.71 | 14.48 |
| SB12 T11 | 3.32 | 0.33 | 5.25 | 1.72 | 0.54 | 3.97 | 14.68 |

| Wine | pH | VA | TA | Malic A | Glucose | Fructose | Ethanol |
|---|---|---|---|---|---|---|---|
| SB12 T12 | 3.29 | 0.33 | 5.16 | 1.72 | 0.92 | 5.26 | 14.44 |
| SB12 T13 | 3.28 | 0.33 | 5.18 | 1.62 | 0.9 | 5.73 | 14.57 |
| SB12 T14 | 3.33 | 0.32 | 5.37 | 2.06 | 0.61 | 2.73 | 14.06 |
| SB12 T15 | 3.19 | 0.31 | 5.51 | 1.85 | 0.98 | 6.89 | 14.2 |
| M10 E1 | 3.44 | 0.46 | 7.52 | 2.83 | -0.82 | 0.92 | 13.76 |
| M10 E2 | | | | | | | |
| M10 E3 | 3.35 | 0.5 | 7.78 | 2.88 | -0.33 | 1.01 | 13.88 |
| M10 E4 | 3.26 | 0.58 | 7.84 | 2.57 | 0.05 | 1.13 | 14.85 |
| M10 E5 | | | | | | | |
| M10 T21 | 3.45 | 0.5 | 7.45 | 2.53 | -0.44 | 1.21 | 14.52 |
| M10 T22 | 3.29 | 0.58 | 7.63 | 2.6 | -0.12 | 0.92 | 14.59 |
| M10 T23 | 3.41 | 0.46 | 7.59 | 2.65 | -0.61 | 0.91 | 13.63 |
| M10 T24 | 3.23 | 0.73 | 7.82 | 2.35 | 0.89 | 1.76 | 16.38 |
| M10 T25 | | | | | | | |
| M10 T11 | 3.51 | 0.42 | 7.25 | 2.43 | -0.55 | 0.99 | 13.92 |
| M10 T12 | 3.44 | 0.55 | 7.48 | 2.61 | -0.44 | 0.93 | 13.97 |
| M10 T13 | 3.22 | 0.65 | 7.89 | 2.37 | 0.47 | 1.31 | 15.67 |
| M10 T14 | | | | | | | |
| M10 T15 | | | | | | | |
| M11 E1 | 3.47 | 0.29 | 6.6 | 1.53 | -0.17 | 0.95 | 14.56 |
| M11 E2 | 3.4 | 0.25 | 7.01 | 2.02 | -0.25 | 0.9 | 14.82 |
| M11 E3 | 3.26 | 0.26 | 7.67 | 2.47 | -0.27 | 0.93 | 14.47 |
| M11 E4 | 3.16 | 0.37 | 7.8 | 2.39 | -0.35 | 0.99 | 14.16 |
| M11 E5 | 3.32 | 0.46 | 7.27 | 2.21 | 0.02 | 1.4 | 15.38 |
| M11 T21 | 3.42 | 0.29 | 6.79 | 1.74 | -0.55 | 0.88 | 14.48 |
| M11 T22 | 3.25 | 0.29 | 7.5 | 2.16 | -0.21 | 1.06 | 14.4 |
| M11 T23 | 3.28 | 0.22 | 7.59 | 2.52 | -0.32 | 1.03 | 14.7 |
| M11 T24 | 3.25 | 0.43 | 7.23 | 1.78 | -0.12 | 1.36 | 13.92 |
| M11 T25 | 3.26 | 0.37 | 7.69 | 2.53 | -0.67 | 0.73 | 13.6 |
| M11 T11 | 3.44 | 0.32 | 6.88 | 1.66 | -0.45 | 1.05 | 14.59 |
| M11 T12 | 3.38 | 0.28 | 7.01 | 2.04 | -0.06 | 1.06 | 14.84 |
| M11 T13 | 3.29 | 0.35 | 7.3 | 1.67 | -0.08 | 1.33 | 15.08 |
| M11 T14 | 3.18 | 0.43 | 7.65 | 2.33 | -0.22 | 0.98 | 14.11 |
| M11 T15 | 3.37 | 0.39 | 6.92 | 1.81 | -0.58 | 1.12 | 13.48 |
| M12 E1_2 | 3.59 | 0.62 | 6.53 | 0.64 | -0.5 | 1.33 | 13.75 |
| M12 E3 | 3.54 | 0.6 | 6.59 | 0.71 | -0.11 | 1.49 | 14.24 |
| M12 E4 | 3.28 | 0.38 | 7.36 | 1.9 | -0.18 | 1.16 | 13.74 |
| M12 E5 | 3.62 | 0.62 | 6.26 | 0.76 | -0.55 | 1.4 | 13.95 |
| M12 T21 | 3.7 | 0.49 | 6.15 | 0.66 | -0.38 | 1.29 | 14.09 |
| M12 T22 | 3.54 | 0.34 | 6.68 | 1.11 | -0.05 | 1.28 | 14.32 |
| M12 T23 | | | | | | | |
| M12 T24 | 3.47 | 0.46 | 6.43 | 0.69 | -0.05 | 1.27 | 13.4 |
| M12 T25 | 3.64 | 0.52 | 6.34 | 0.55 | -0.17 | 1.08 | 13.75 |
| M12 T11 | 3.74 | 0.48 | 5.87 | 0.64 | -0.12 | 1.39 | 14.82 |

| Wine | pH | VA | TA | Malic A | Glucose | Fructose | Ethanol |
|---|---|---|---|---|---|---|---|
| M12 T12 | 3.53 | 0.43 | 6.71 | 1.13 | 0.08 | 1.47 | 14.66 |
| M12 T13 | 3.45 | 0.32 | 6.83 | 0.85 | -0.35 | 1.34 | 13.97 |
| M12 T14 | 3.36 | 0.31 | 7.54 | 2.78 | 0.09 | 1.16 | 13.94 |
| M12 T15 | 3.68 | 0.47 | 6.1 | 0.8 | -0.5 | 1.3 | 13.64 |
| M12 P1_2 | 3.7 | 0.76 | 6.24 | 0.6 | -0.98 | 1.46 | 13.77 |
| M12 P3 | | | | | | | |
| M12 P4 | 3.48 | 0.35 | 7.21 | 2.32 | 0.06 | 1.45 | 14.43 |
| M12 P5 | 3.67 | 0.56 | 6.17 | 0.74 | -0.51 | 1.45 | 13.56 |