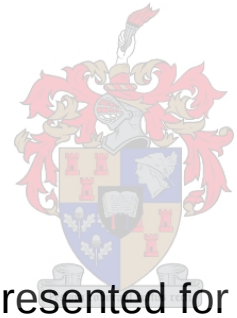
The effect of a creosote stockyard on the environment, vines and wines

by

Annette van Zyl



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Declaration

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Summary

The quality of wine is largely dependent on environmental conditions and recent studies have therefore focused on agricultural practices in terms of water, soil and biodiversity conservation. The industry aims to create sustainable practice and to protect the natural resources available. Sources of potential grape contamination include: vehicle pollution, pesticides, bushfires and wood preservatives used for trellising systems. The latter have come to the attention of the South African wine industry (e.g. creosote and Copper Chromium Arsenate (CCA) products) as they may have consequences for the environment and wine quality. Creosote is a known pollutant of soil and ground water and the volatile fraction has been monitored in air. Plants may also accumulate polycyclic aromatic hydrocarbons (PAHs), which constitute up to 85% of the mass of creosote, and of which some have been proven carcinogenic. Because of the health risks associated with it, creosote has therefore been restricted for use in most applications in Europe, and also in the United States, Canada and Australia.

This study focussed on the sensorial and chemical analyses of environmental and wine samples taken from the area around the creosote stockyard to determine accumulation of creosote-derived compounds. Environmental samples were collected and analysed at different distances from the affected area, over two vintages. Wines were made from grapes grown in vineyard blocks adjacent to the stockyard, to determine the effect of distance and skin contact during alcoholic fermentation treatments on wine taint. A sensory panel was trained for descriptive analysis to determine the intensity of the taint. Analytical methods were developed for the analysis of volatile organic compounds (VOCs) by gas chromatography mass spectrometry (GC-MS) and polycyclic aromatic hydrocarbons by high performance liquid chromatography with diode array detection (HPLC-DAD).

The sensory results obtained showed white and rosé wines were perceived as clean, whilst the red wines were associated with burnt rubber/tar taint. The perceived taint decreased as distance from the stockyard increased. Wines made from the Cabernet Sauvignon blocks adjacent to the stockyard also showed an increase of taint with the increase of skin contact. Chemical data obtained showed that the taint consisted of a complex mixture of compounds, each with its own pattern of retention within the vineyard and wine. Only m-cresol was found above odour threshold, and only in red wines. The synergistic effect of some compounds may lead to an increase in the perceived taint.

Berries and leaves had higher concentrations of volatile compounds than wines. Leaf contamination varied and followed the general trend in literature where the plants with high lipid content and exposed leaf area were the most contaminated. There may be other

compounds present in creosote emissions, aside from those investigated here, with sensory attributes related to the taint found in wines. These compounds are styrene, indene, benzene, toluene, isoquinoline and quinoline and should be included in further investigations.

From the PAH analysis of environmental samples and wines, it is evident that the samples closest to the stockyard were affected the most. The contamination varied with the depth of the soil: some of the heavier compounds were found in the samples taken from the deeper levels, whilst nearly all other target compounds were present in the top layer of soil. The concentrations found in the environmental samples were lower than reported in literature. Wines had few PAHs present, but at much higher concentrations than is allowed by EU legislation.

From this study it is evident that the stockyard had negative effects on the surrounding environment in terms of sensory and chemical contamination. Recommendations include area rehabilitation by means of bioremediation to protect resources and ensure sustainable and safe production of crops. Industrial emissions should also be regulated and restricted in agricultural areas. Furthermore the use of creosote should be revised, and prohibited for agricultural use.

Opsomming

Die kwaliteit van wyn is grootliks afhanklik van die omgewingstoestande. Daarom fokus verskeie onlangse studies op landbou aktiwiteite en die invloed daarvan op die omgewing in terme van water, grond en biodiversiteit bewaring. The wyn industrie se doelwitte sluit volhoubare praktyke in, wat die natuurlike hulpbronne bewaar en beskerm. Druiwe kan deur middel van die volgende bronne besoedel word: brandstof uitlaatgasse, insekdoders, veldbrande, gifstowwe wat gebruik word vir houtperservering wat dan verder aangewend word vir opleistelsels. Houtperservering (Koper chroom arsenaat (CCA) en kreosoot) het veral in die laaste paar jaar onder aandag gekom in die wynbedryf van Suid-Afrika as gevolg van die invloed daarvan op die omgewing en die uiteindelike wynkwaliteit. Kreosoot is ‘n gekende gifstof wat verantwoordelik is vir grond en grondwater besoedeling en wat gemonitor word in die atmosfeer. Plante akkummuleer ook poli-sikliese aromatiese hidro-koolstowwe (PAHs), wat to 85% uitmaak van die massa van kreosoot. PAHs is karsinogenies en dus is daar baie navorsing op die molekules reeds gedoen. Die gesondheidsrisikos gepaardgaande met kreosoot het gelei tot die streng geregulasies tans ingestel in Europa, die Verenigde State, Kanada en Australië.

Hierdie studie het gefokus op die sensoriese en chemiese analises van omgewings- en wyn-monsters geneem van die omliggende area van die kreosoot palewerf om die akkumulasie van kreosoot-afgeleide-verbindings. Omgewingsmonsters was versamel en geanaliseer om verskillende afstande vanaf die bron van besoedeling (palewerf) te ondersoek oor ‘n twee jaar periode. Wyne is gemaak van die druiwe wat afkomstig is van die blok aangeplant langs die palewerf. Die wyne is ondersoek in terme van afstand vanaf die kreosoot bron asook oenologiese invloede, dopkontak gedurende alkoholiese fermentasie, op die kontaminasie beskryf in wyn. Die wyne is ook oor ‘n twee jaar periode voorberei en sluit die 2011 en 2012 seisoen in. ‘n Sensoriese paneel is opgelei om die beskrywende analises op die wyn uit te voer met die doel om die intensiteit van kontaminasie te identifiseer. Analitiese metodes is ook ontwikkel vir die analise van vlugtige organiese verbindings(VOCs) met gas chromatografie-massa spektrometrie (GC-MS) en poli-sikliese aromaties hidro-koolstowwe (PAHs) met hoë druk vloeistof chromatografie.

Die sensoriese resultate bekom het wit en rosé wyne as skoon laat blyk, terwyl rooi wyne meer geassosieer was met die gebrande rubber/ teer afgeur beskrywing. Die waargeneemde afgeur het afgeneem soos wat die afstand vanaf die palewerf toegeneem het. Wyne gemaak van die Cabernet Sauvignon blokke langsaan die palewerf het ook ‘n toename in die afgeur gehad met ‘n toename in dopkontak. Chemiese data bekom beeld

uit dat die afgeur uit ‘n komplekse mengsel van verbindings bestaan, elk met sy eie patroon van verspreiding en verbinding in die wingerd as ook in die wyn. Net m-kresol was gevind bo die reuk drumpel, dit het ook net in rooi wyne voorgekom. Die sinergistiese effek van die verbindings mag egter bydra tot die waargeneemde afgeur.

Druiwekorrels en blare het hoër konsentrasies van die vlugtige verbindings gehad as wat gemeet is in die wyne. Blaar kontaminasie het ook baie gewissel en het ooreengestem met die algemene tendens wat in literatuur beskryf is, naamlik dat plante met ‘n hoër lipid inhoud en grootter blaar oppervlak die meeste gekontamineer word. Daar mag egter nog baie ander verbindings bydra tot die waargeneemde afgeur gevind in die wyn. Spesifieke verbindings wat wel ‘n rol kan speel in kontaminasie en wat voorkom in die vlugtige gedeelte van kreosoot is styreen, indeen, benzeen, tolueen, isoquinoleen die vlugtige verbindings van kreosoot. Die verbindings moet ingesluit word vir verdere studies wat gedoen word op die kreosoot geassosieerde afgeur.

Die PAHs analise op die omgewingsmonsters en wyne het gelei tot die bevestiging dat die naasliggende omgewing die meeste geaffekteer is. Die kontaminasie wissel in terme van die diepte in die grond wat die gifstowwe voorkom: die swaarder molekulêre verbindings is tot in die dieper vlakke waargeneem terwyl al die gemete verbindings in die boonste lae teenwoordig was. Die vlakke wat waargeneem is in dié studie is egter laer as wat voorheen in literatuur gevind is in ‘n kreosoot geaffekteerde omgewing. Wyn het PAHs teenwoordig gehad, alhoewel slegs twee verbindings gemeet is, het dit in hoër vlakke voorgekom as wat sekere Europese regulasies as toelaatbaar spesifiseer.

Vanaf die studie resultate blyk dit, dat die palewerf se negatiewe invloed op die omliggende omgewing beide meetbaar was in sensories en chemiese kontaminasie. Voorstelle sluit onder andere die rehabilitasie van die omliggende omgewing deur middel van bioremediasie in. Om sodoende die natuurlike hulpbronne in die area te bewaar asook om volhoubare en veilige verbouing van gewasse te verseker. Industriële besoedeling en afval moet ook gereguleer word en beperk word in landbou areas. Verder moet die gebruik van kreosoot heroorweeg word en strenger regulasies moet in plek gestel word om aan internastionale standaarde te voldoen.

Biographical sketch

Annette van Zyl was born on 29 June 1988. She attended C&N Meisieskool Oranje in Bloemfontein and matriculated in 2006. Thereafter she enrolled for a BScAgric-degree at the Stellenbosch University, majoring in Oenology Specialised. She completed the Bachelors degree in 2010 and a harvest internship at Neil Ellis Wines. Subsequently this MSc research was started in 2011 and completed in 2012. In 2012 she enrolled for Wine marketing course in Agricultural economics and continued work in the industry through-out her studies.

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Preface

This dissertation is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the South African Society of Viticulture and Enology.

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** The impact of creosote on the environment and agriculture and the chemical analysis of samples.
Chapter 3	**Research results** The environmental impact of PAHs on soil and water from an industrial source situated in an agricultural area.
Chapter 4	**Research results** Monitoring chemical and sensory effects of environmental contaminants on vine and wine.
Chapter 5	**Conclusion**

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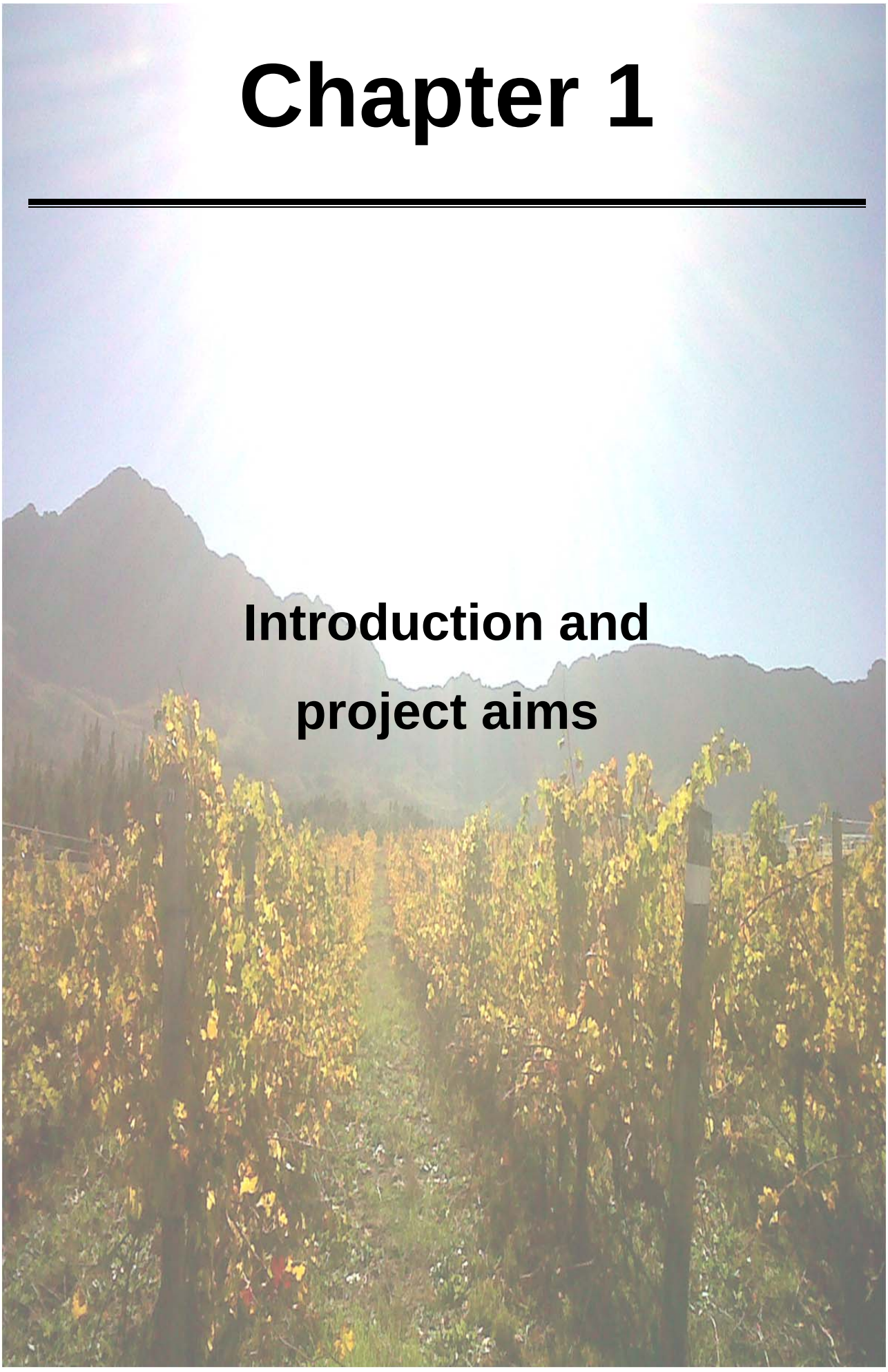
Chapter 1



Introduction and project aims

1.1.1 INTRODUCTION

The South African wine industry is situated mainly in the Western Cape, an area faced with many challenges regarding conservation and pollution. Due to the nature of the international wine market, the environmental impact of farming activities is becoming vital for a sustainable industry. There are many challenges faced by producers to export markets, as countries often differ in legislation (Wenzl, *et al.*, 2006). It is important for producers to consider the most sustainable practices in terms of their environment and workers, as these aspects are becoming increasingly important for the consumer. In South Africa one of the current concerns is the use of creosote poles in the vineyard, as many studies have shown the environmental impact of creosote, mainly due to leaching in soil and groundwater. PAHs constitute 85% of creosote components, 10% phenolic compounds and 5% N-, O-and S-heterocyclic compounds (Meyer, *et al.*, 1999).

PAHs are organic compounds containing two or more fused benzene rings. They are carcinogenic and mutagenic compounds which have been extensively researched for their impact on human health, food contamination and their role as organic pollutants in the environment (World Health Organization, 2004). Both creosote and PAHs have been legislated and even prohibited in some areas, for example, in the European Union and the United States of America (Mateus, *et al.*, 2008).

In agriculture, experiments have been conducted worldwide over recent years for determination of both creosote and PAH contamination. Impact studies have been carried out on vegetables, olive oils, soil, air and water (Hale & Aneiro, 1997; Kipopoulou, *et al.*, 1999; Meyer *et al.*, 1999; Becker, *et al.*, 2001; Toa, *et al.*, 2006; Moret, *et al.*, 2007; Gallego, *et al.*, 2008). The mechanisms of plant uptake of PAHs have also been investigated (Simonich & Hites, 1995; Wang, *et al.*, 2010). Furthermore, various methods for extraction of compounds of interest and analysis by gas chromatography (GC) (Eriksson, *et al.*, 2001; Poster, *et al.*, 2006) or liquid chromatography (LC)(Camargo & Toledo, 2003; Moret, *et al.*, 2007) are available in literature. Standard methods are in place for analysing these compounds in soil sediments and drinking water, amongst others, as set up by the Environmental Protection Agency (EPA) (Poster, *et al.*, 2006). Methods differ depending on the environmental sample and the matrix involved. Alternatives for creosote include various products, e.g. metal stakes, recycled plastic poles, and Tanalith® treated poles, but are country specific. Bioremediation (Atagana, H.I., 2004; Miller, *et al.*, 2004), vermi-remediation (Sinha, *et al.*, 2008) and compost studies have been investigated as methods for rehabilitation of contaminated soils and waste materials, respectively.

This project aims to investigate to the impact of a creosote stockyard situated in an agricultural area in South Africa on vine and wines. This study was part of a bigger research project which investigated the effects of different trellising systems on wine quality, as well

as alternative sources of PAHs in the vineyard and the uptake of PAHs in the vine and into wines. These studies will provide new insight into the behaviour of the carcinogenic PAHs and volatile compounds in wine.

1.1.2 Vineyard background and problem statement

Lourensford Estate is situated at the foot of the Helderberg Mountains on a 4500ha agricultural farm. Conservation attempts by Biodiversity Wine Initiative (BWI), a programme of the World Wildlife Fund, are currently part of their environmental program to preserve the Cape Floral Kingdom (CFK) heritage. Various practices, e.g. alien species removal and natural resource protection, are part of this initiative. The farm is host to various industrial activities, including a creosoting plant (now closed), where wooden posts were treated with creosote and stored in an open storage area to dry. During drying, the freshly creosoted posts emit phenolic compounds and volatile PAHs into the atmosphere. These emissions are easy to detect as they have a very powerful, tarry odour that permeates a large area surrounding the stockyard. The natural habitat around the area, which includes vineyards and orchards, is therefore exposed to these organic pollutants. From literature it is evident that creosote has detrimental consequences for the environment, which includes pollution of soil (PAHs) (Eriksson, *et al.*, 2001), water (lower molecular PAHs) (Hale & Aneiro, 1997) and air (volatile fraction) (Mateus, *et al.*, 2008). Studies have shown that PAHs sourced from creosote are taken up by terrestrial plants and can accumulate on plants surfaces grown in polluted areas (Moret, *et al.*, 2007; Wu, *et al.*, 2008) This study investigated the environmental repercussions of the stockyard in terms of PAH accumulation in soil, water and leaf samples as well as the effects on quality of wines. The wines made from exposed vineyard blocks had a complex taint described as burnt-rubber/tar and would therefore not be suitable for sale to markets. The creosote plant was closed in 2010, but the stockyard remained in use until the end of 2011. From 2012 the plant was replaced with timber manufacturing industry using safer alternatives to creosote.

1.1.3 Project Aims

This study aimed to:

i. Analyse volatile phenols and polycyclic aromatic hydrocarbons in biological samples taken from around a creosote stockyard, using GC-MS and HPLC.

ii. Investigate the impact of the creosote stockyard on accumulation of PAHs and volatile phenols in water, leaf and soil samples from the immediate environment.

iii. Investigate the impact of volatile compounds / taint derived from creosote on wines that were made from grapes grown in vineyard blocks adjacent to the stockyard using sensory analysis.

iv. Clarify the role of winemaking practices on concentration and/or decreasing the taint in affected wine.

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Chapter 2

Literature review

2.1 INTRODUCTION

At the present time the use of creosote as wood preservative is still fairly common in the South African agricultural sector. Creosote is a distillation product of crude tar oil, with a typical sharp smoky, tarry odour. Its chemical composition contains up to 75 - 85% polycyclic aromatic hydrocarbons (PAHs), 10% phenolic compounds and 5% N-, S-, O-heterocyclic compounds, depending on the distillation conditions (Mateus, *et al.*, 2008). PAHs and creosote have been extensively researched over the last few decades, and studies have shown that these compounds have health implications for both biological systems and the environment. This has led to usage restrictions in the US, EU, Australia and Canada. Previous studies have also researched the volatile fraction emitted from creosote (Gallego, *et al.*, 2008), the health implications for workers and residents living in a neighbourhood nearby a creosote plant (Dahlgren, *et al.*, 2003), the fate of creosote in the environment (Hale & Aneiro, 1997; Meyer, *et al.*, 1999; Becker, *et al.*, 2001; Eriksson, *et al.*, 2001; Gallego, *et al.*, 2008) and the contamination of produce, including olive oil from a grove close to railway ties (Moret, *et al.*, 2007). PAH studies have theorised pathways of plant uptake of atmospheric pollutants (Simonich & Hites, 1995), contamination of vegetables (Tao, *et al.*, 2005) and (olive) oils (Moret, *et al.*, 2007). The phenolic fraction, e.g. cresols, phenols and naphthalene, have also been studied in water, but are not always directly connected with creosote, as they can originate from various sources, e.g. pesticides (Asan & Isildak, 2002). This chapter aims to overview creosote use in the agricultural sector with research studies available from literature.

2.2 CREOSOTE

2.2.1 Chemical properties

Creosote is a very complex by-product of coal-tar oil distillation, and consists of more than 300 derivative compounds (Mateus, *et al.*, 2008). Creosote is applied to preserve wood used mainly in the construction and communication industries for railway ties, electrical power poles, marine pilings, and fences. It is used for its fungicidal, insecticide and water repellent properties, which are the main causes of wood deterioration (Competent Authority Report, 2007). It is sometimes used for specialised applications for instance children's playgrounds, and also agricultural structures, e.g. trellising systems for fruit production. Derivative compounds form part of four chemical groups: PAH, N-, O- and S containing hetero-PAH, phenolic compounds and mono-aromatic hydrocarbons (Meyer, *et al.*, 1999). Classification of creosote is due to differences in the distillation process and the origin of the

coal. Creosote is classified by boiling points of ≥210-400˚C and ≥260-400˚C and is classified grade B and grade C respectively by the Competent Authority (CA) for the European Commission (EC). Grade B is mainly used for wood preservation, although grade C is allowed since it has a lower volatility. Creosote is denser than water, and soluble in organic solvents e.g. benzene, toluene, acetone and quinoline. Creosote is considered to be stable, not affected by pH and not flammable, although emissions may cause explosive mixtures with air.

Health risks associated with creosote are usually due to exposure to PAHs and/or volatiles. Humans are exposed to these health threats by breathing in volatile fractions and consumption of water or foodstuffs containing PAHs. Higher molecular weight PAHs are carcinogenic and mutagenic (Gallego, *et al.*, 2008; Moret, *et al.*, 2007). Environmental concerns include soil, water and air pollution. Different authors have found residues or accumulation of these hazardous compounds in foodstuffs (Kipopoulou, *et al.*, 1999; Tao, *et al.*, 2005; Wang, *et al.*, 2010); although few of these studies looked at creosote as the source of the PAHs.

Table 1: Major components of creosote as compiled by the International Uniform Chemical Information Database (IUCLID) (Gallego, *et al.*, 2008).

Table 1 – Major components in creosote

Compound	CAS number	Percentage (%)
Naphthalene	91-20-3	<1–10
1-/2-Methylnaphthalene	90-12-0/91-57-6	2–18
Indene	95-13-6	<1
Fluorene	86-73-7	1–7
Acenaphthylene	208-96-8	<1
Acenaphthene	83-32-9	1–8
Phenanthrene	85-01-8	5–20
Anthracene	120-12-7	<1–3
Fluoranthene	206-44-0	4–15
Pyrene	129-00-0	2–10
Benzo[*a*]anthracene	56-55-3	<0.1
Chrysene	218-01-9	<0.1
Benzo[*a*]pyrene	50-32-8	<0.005
Benzo[*b*]fluoranthene	205-99-2	<0.025
Benzo[*k*]fluoranthene	207-08-9	<0.025
Indeno[1,2,3-*cd*]pyrene	193-39-5	<0.025
Dibenzo[*a*,*h*]anthracene	53-70-3	<0.025
Benzo[*g*,*h*,*i*]perylene	191-24-2	<0.025

[a] IUCLID (2002).

The volatile fraction of creosote consists mostly out of the less polar volatile organic compounds (VOCs) with one benzene ring, naphthalene being the most abundant as illustrated in Table 1. Lower emission rates were observed for the 2 – 4 ringed PAHs. The

polar heavy PAHs, 5-ring structures, where not present in the volatile fraction. Naphthalene, 2-methylnaphthalene, toluene, m + p-xylenes, ethyl benzene, o-xylene, iso-propyl benzene, benzene and phenol are the most abundant in newly treated poles (Gallego, *et al.*, 2008).

2.2.2 Legislation

The US-Food & Drug Administration (FDA) and Nationals Oceanic and Atmospheric Administration (NOAA) are the major legislative authorities for petroleum hydrocarbons in environmental matrixes. The substance is regulated by national legislation which is different for each country. The South African Wood Preservers Association (SAWPA) is responsible for industry standards and guidelines to abridge the risks associated with exposure of treatment plant workers and environments to creosote chemicals. Creosote has been classified as a probable human carcinogenic and hazardous substance by the European Union (EU), United States (US-EPA); The Agency for Toxic Substances and Disease Registry (US-ATSDR)), Australia (The National Health and Safety Commission (NOHSC) and Canada (Mateus, *et al.*, 2008). Phenanthrene has been identified as the predominant PAH in creosote, followed by naphthalene and fluoranthene. This study also found benzo(a)pyrene concentrations of 544 – 4432 ppm in creosote treated wood used for railroad ties. Due to such high concentrations and the potential health threat associated with PAHs, the EU adopted a Directive that only allows creosote treated wood to have 50 ppm benzo(a)pyrene content for industrial application. Legislation recommends regular quality assessments of air, soil, water and tissue in these affected areas (Moret, *et al.*., 2007).

The US- Environmental Protection Agency (EPA) and EU have defined 16 PAHs as priority pollutants. The PAHs were selected on their carcinogenic properties, and is often expressed in benzo(*a)*pyrene equivalents.

Table 2: Priority PAHs as defined by environmental and health studies.

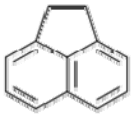
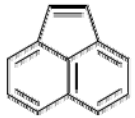
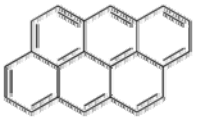
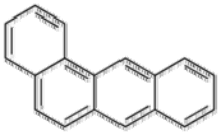
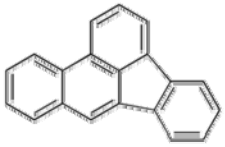
World Health Organization Environmental health criteria	Structure	Genotoxicity	Carcinogenic	US EPA	Creosote constituents [a]	Concentration in wine [b]
Acenaphthene		(?)	(?)	X	X	
Acenaphthylene		(?)	no data	X		
Anthanthrene		(+)	+			
Anthracene		-	-	X	X	
Benz[a]anthracene		+	+	X	X	
Benzo[b]fluoranthene		+	+	X		2ng/L = 0.002ppb

Table 2 (cont.)

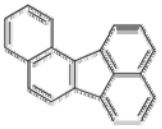
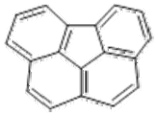
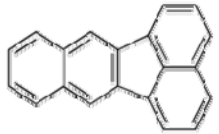
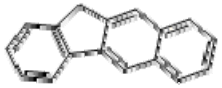
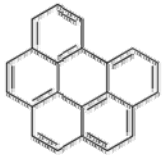
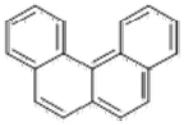
Benzo[j]fluoranthene		+	+		
Benzo[ghi]fluoranthene		(+)	(-)		
Benzo[k]fluoranthene		+	+	X	2ng/L = 0.002ppb
Benzo[a]fluorene		(?)	(?)		
Benzo[b]fluorene		(?)	(?)		
Benzo[ghi]perylene		+	-	X	
Benzo[c]phenanthrene		(+)	+		

Table 2 (cont.)

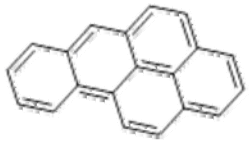
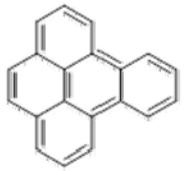
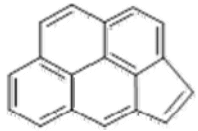
Benzo[a]pyrene		+	+	X	X	6ng/L = 0.006ppb
Benzo[e]pyrene		+	?			
Chrysene		+	+	X	X	
Coronene		(+)	(?)			
Cyclopenta[cd]pyrene		+	+			

Table 2 (cont.)

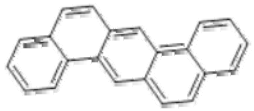
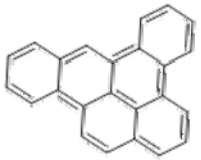
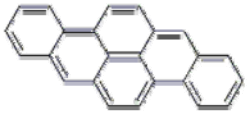
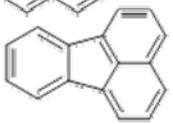
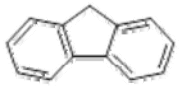
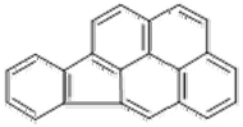
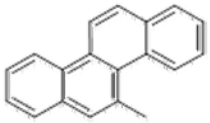
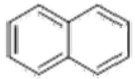
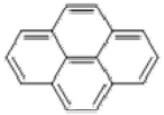
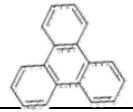
Dibenz[a,h]anthracene		+	+	X	
Dibenzo[a,e]pyrene		+	+		
Dibenzo[a,h]pyrene		(+)	+		
Dibenzo[a,i]pyrene		+	+		
Dibenzo[a,l]pyrene		(+)	+		
Fluoranthene		+	(+)	X	X
Fluorene		-	-	X	X

Table 2 (cont.)

Indeno[1,2,3-cd]pyrene		+	+	X		0.06ppb
5-methylchrysene		+	+			
1-methylphenanthrene		+	(-)			
Naphthalene		-	(?)	X	X	
Perylene		+	(-)			
Phenanthrene		(?)	(?)	X	X	
Pyrene		(?)	(?)	X	X	
Triphenylene		+	(-)			

(a) Gallego, *et al.*, 2008 (b) Chatonnet & Escobessa, 2007. Coding: + = positive, -=negative,? = questionable, parentheses indicates small database.

The adapted Table 2 indicates the 16 priority PAHs recognised as a potential threat to the environment and humans by the US-EPA, as well as their carcinogenic nature (Poster, *et al.*, 2006). The chemical structures are included, showing the 2-5 ring structures as well as the PAHs that form the major constituents of creosote (Table 1 and 2).

In South Africa the industry of wood preservation is guided by SANS 100005 and SANS 457 laws, neither of which has restrictions regulating the use of creosote. A common application of creosote in South Africa is the constructed trellising systems, e.g. in vineyards. The Integrated production of wine (IPW) regulates the sustainability of practices in the wine industry, advises producers to use alternatives to creosote, but no compulsory regulations were in place by the time this research was done. Creosote alternatives will be discussed in further detail under the discussion section.

2.3 Environmental pollution

Creosote poses a major environmental threat, as the constituents, (particularly the PAHs) are pollutants in the natural environment. Therefore the environmental impact of creosote is often assessed in total PAH concentration (ATSDR, 2003). The biggest impact is on soil, water and air; however these components form the habitat of fauna and flora and therefore the contamination could spread to plants or animals. PAH uptake in plants are ascribed to the fat content or log octanol-water coefficient (K_{OW}) properties of the plants, location of the growing sites and the surface area exposed to air pollution. Humans are exposed to the toxic vapours emitted from the creosote-treated wood via the air; however workers can be in direct contact during application and installation of treated wood. Particular-bounded PAHs can be another potential source of contaminants, exposing near-living residents to dust-bounded PAH (Dahlgren, *et al.*, 2003).

Creosote leaches into the soil forming ‘plumes’, due to rainwater/irrigation leaching or oil exudation. Total PAH concentrations in storage areas of treated wood can accumulate in soil up to concentrations of several thousand ppm in dry weight. The molecules that leach into soil are usually the bigger molecular weight molecules, as the smaller molecular weight compounds are highly volatile. Heavy PAHs, classified by the number of aromatic rings, can persist in soil for years, as they are not degraded biotically or abiotically (Moret, *et al.*, 2007). A number of studies have been carried out in order to assess the effects of creosote in the environment. Creosote-contaminated soil from an old gas work site in Sweden was researched (Eriksson, *et al.*, 2001). The authors firstly classified the soil as sandy, 0.1-5 mm, with low organic material content and < 10% water content. They found PAHs concentrations from below detection up to 5000 ppm in soil sampled from the site. Greek authors found that seasonal effects played a significant role in the distribution of PAHs in

soil, this was seen as lower concentrations of the 2-3 ring PAHs (Acenapthene, Fluorene and Phenanthrene) were observed in the soil of industrial areas. Polycyclic aromatic hydrocarbons can be lost from soil due to leaching, uptake by plants (Kipopoulou, *et al.*, 1999), volatilization (temperature increase), and abiotic (sorption and volatilization) and biotic degradation (microbial and solubility) (Miller, *et al.*, 2004). Photochemical reactions may degrade PAHs in soil, but are restricted to the top layer of soil. Season effects that play a role are: rainfall and temperature increases. After winter rainfall, the PAHs from industrial or urban areas may leach into the soil. During seasonal temperature increases, higher volatilization may take place, leading to lower concentrations of the lower molecular weight PAHs (Kipopoulou, *et al.*, 1999).

Water sources may be directly contaminated via the wood preservative plant, or can be polluted via soil leaching. The lower molecular mass compounds that are derived from creosote are water soluble, e.g. naphthalene, and can therefore be found in rivers and groundwater (Hale & Aneiro, 1997; Wenzl, *et al.*, 2006). Water sampling and analysis of potential contaminant sites are vital. Various methods, including an EPA method, have been developed for the analysis of PAHs in natural water (García-Falcón, *et al.*, 2004).

Pollutants in water are regulated by the EU and maximum limits for PAHs in drinking water and foodstuffs are established, although the limits for foodstuffs may vary between EU countries. The maximum allowable benzo(a)pyrene level in drinking water is 0.01 ppb, this represents the sum of benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*g,h,i*)perylene and indeno(*1,2,3-cd)*pyrene (Wenzl, *et al.*, 2006). Phenolic compounds, o-cresol, m-cresol, p-cresol, phenol, resorcicol, catechol and hydroquinone, have also been investigated in water. These compounds, especially the cresol and phenols are commonly found in wood preservatives such as creosote. Asan & Isildak, (2003) found 3.6, 2.2 and 7.6 ppb of phenol, o- and m-cresol respectively in water.

Studies have estimated that 1-2% of creosote used as wood preservative will be emitted into the atmosphere (Mateus, *et al.*, 2008). Atmospheric PAHs can either be classified in the particulate or gaseous phase; the airborne particles are emitted according to the atmospheric conditions (Manoli, *et al.*, 2004). Higher ambient temperature will increase the volatile fraction. Measuring the PAH level in air is an important part of air-quality monitoring (Poster, *et al.*, 2006).

Studies have shown that passive sampling of PAHs in air is enhanced if there is movement of the air, therefore the wind will play an important part in the distribution of the compounds. The compounds physicochemical properties will also play a role, compounds with a higher log octanol-air coefficient (K_{OA}) are restricted by the boundary layer (of air) (Söderström & Bergqvist, 2005).

Table 3: Emission of compounds from newly treated creosote posts (Gallego, *et al.*, 2007)

Table 2 – Emission concentrations ($\mu g\,m^{-3}$) ± RSD (relative standard deviation, %) evolution according to the days of residence of the treated wood in the field storage

Id. Number	Compound	Days of residence				
		1	2	5	6	8
1	Naphthalene	24059±22	12809±11	6310±6	3556±29	3324±3
2	Toluene	9856±73	1678±14	499±10	301±28	431±5
3	*m*+*p*-Xylenes	8143±82	7667±11	1016±15	486±21	659±1
4	Ethylbenzene	7464±65	1584±12	563±12	295±25	394±1
5	o-Xylene	2800±61	784±11	296±13	146±19	205±2
6	Isopropylbenzene	2440±54	936±11	403±17	198±17	275±14
7	2-Methylnaphthalene	2248±87	575±34	555±26	143±24	248±1
8	Benzene	2218±61	476±17	137±7	111±32	104±27
9	Phenol	1079±25	1284±13	1834±24	1131±14	2257±6
10	Biphenyl	845±19	472±6	576±15	354±25	378±2
11	Acenaphthene	681±100	1112±5	1028±9	647	139±58
12	Benzonitrile	654±38	371±17	220±15	104±18	193±18
13	2,6-Dimethylnaphthalene	109±18	62±26	58±2	43±16	38±3
14	1-Methylnaphthalene	94±34	58±16	67±13	36±19	57±7
15	Benzofuran	90±45	42±14	20±15	10±20	15±7
16	Fluorene	79±81	113±3	133±31	81	20±25
17	1,2-Dimethylnaphthalene	78±33	47±26	57±4	40±18	33±12
18	1-Ethylnaphthalene	63±14	39±13	34±15	25±4	27±7
19	Propenylbenzene	55	27±1	11±18	3.6±3	2.9±10
20	Benzo[*b*]tiophene	17±35	9.0±9	5.2±19	3.8±11	3.9±10
21	Acenaphthylene	4.8±83	8.6±11	8.4±25	4.0	0.8±25
22	Anthracene	1.4	2.6	3.3±33	4.2	6.2
23	Pyrene	1.20±2	0.30±7	0.97±18	0.76	1.22±2
24	Indene	0.32±3	0.37±11	0.36±14	0.17±24	0.20±15

Emission rates of PAHs, due to creosote field storage, were found to be 4 – 28 $\mu g.m^{-3}$ whilst the volatile organic compounds (VOC) ranged for 5 - 35 $mg.m^{-3}$. This was measured over an 8 day period after creosote treatment, concentrations decreased from highest parameter to lowest during this time. Naphthalene was emitted at the highest concentrations, which corresponded with the findings of other authors (World Health Organization (WHO), 2004). Other compounds that were recorded in abundance were toluene, o-, m- and p-xylenes and ethylbenzene, phenol (Table 3). These compounds were mostly VOCs with one benzene ring, larger PAHs were emitted in lower concentrations, and the 5-ring PAHs were detected only in the creosote particulate emission (Gallego, *et al.*, 2008).

Vegetation is potentially exposed to creosote derived pollutants via the air, soil and water. Various pathways exist through which the organic pollutants may enter the plant. Uptake from contaminated soil surrounding the root system and translocation into the plant by the xylem and gas or particulate deposition onto the wax cuticle of the leaves and translocation through the stomata and phloem are two such pathways. The pathway of distribution depends on the chemical nature of the molecules: lipophilicity, water solubility and vapour pressure. Environmental conditions, e.g. temperature and organic content of the

soil as well as the plant species, area and lipids available, will also play a role in the uptake (Simonich & Hites, 1995). The organic content of the soil has adsorption properties on the PAHs, decreasing the mobility in soil (Moret, *et al.*, 2007). The accumulation of PAHs in plants is important for determination of the safety of these potential foodstuffs, as this is the considered as the main source of human exposure to harmful PAHs.

2.4 Agricultural produce

Although creosote uses are restricted in most first-world countries (EU, US, Canada, Australia), applications in the agricultural industry still occur in some areas in New Zealand, Australia and South Africa. In Australia and New Zealand the use of creosote has to be justified regarding no contamination of crop and soil (Conradie, 2011). Since the 1970's various authors have researched PAHs, pesticides, VOCs and other pollutants that could potentially contaminate foodstuffs (Simonich & Hites, 1995; Wenzl, *et al.*, 2006). Foodstuff may be contaminated during production practises or environmental pollution (Wenzl, *et al.*, 2006). The sources of the PAHs and VOCs vary from vehicle emissions, bushfires, creosote emissions, and waste water irrigation. With analysis of variance (ANOVA), species and season were identified as the most significant contributors to vegetable and soil PAH accumulation, respectively (Kipopoulou, A.M., *et al.*, 1999).

Different chemical wood treatments were investigated in California during 1960-1963. This study aimed to observe the phytotoxic effect on young grapevine development. During this study creosote, E-salts (chromated copper arsenate, CCA), C-salts (ammoniacal copper arsenite) and pentachlorophenol showed to be the most damaging when in contact with a vine (Neubauer & Kasimatis, 1966). This study focused on direct contact of the vine with a treated pole during early growth and signs of health decay, growth inhibition and even death of vines where observed.

Studies by Tao, *et al.* (2006) on cabbage showed a positive correlation between gas or particulate PAHs in air and the PAHs found in the part of the vegetable exposed to the atmosphere. The samples, cabbage, soil and gas and particulate air, were taken from two sites, A and B, site B was a typical example of a farm irrigated with wastewater. Site A was not irrigated with waste water. Higher concentrations of PAHs were found in the cabbage from site B. Although lower levels of pollutants were found in the samples from site A, there was correspondence between the sites in terms of the PAH compounds found. This may conclude that the source of pollutants could be the same. The soil samples' contribution to the accumulated PAHs in the cabbage was insignificant, as it did not fit into the pollution model (Tao, *et al.*, 2006).

These findings were also confirmed by another study conducted by Chinese authors, Wang, *et al.*, in 2010, who found atmospheric deposition to be the main contributor to PAH uptake by vegetables in urban areas. This study investigated six types of vegetables, Chinese cabbage (*Brassica rapa pekinensis*), leaf lettuce (*Lactuca sativa*), leek (*Allium tuberosum*), radish (*Raphanus staivus*), cauliflower (*Brassica oleracea*) and rape (*Brassica campestris*) grown in urban areas. Samples were taken from 23 sites, 21 of these sites were irrigated with waste water from a river. All the samples, except 16% of the Chinese cabbage from this study, were declared safe for consumption. The edible part of the vegetable samples were analysed for the 16 priory PAHs as defined by the EU and US-EPA and concentrations ranged from 158 to 995 ppb (Wang, *et al.*, 2010).

The occurrence of lower molecular weight PAHs, 2 - 3 ringed structures have been found by many authors in vegetables (Kipopoulou, *et al.*, 1999; Carmago & Toledo, 2003; Toa, *et al.*, 2008; Wang, *et al.*, 2010). Greek researchers also found that the dominant pathway for PAHs accumulation was deposition from the vapour phase onto vegetables grown in polluted industrial areas. The emission sources of the pollutants were 1-2 km from the samples taken and included petrochemical industries, tyre production, oil refining, metal smelting and painting amongst other industrial activities. The vegetables investigated were cabbage (*Brassica oleracea capitala*), carrot (*Allium porrum),* lettuce (*Lactuca* sativa), leek (*Allium porrum*) and endive (*Chichhorium endicia*). Lettuce and endive had the highest sum of PAHs, possibly due to the bigger exposed leaf area. The authors found the range of total PAHs were 25-294 ppb in the vegetables studies. The solubility and octanol-water partition coefficient of PAHs were found to be associated with the soil-to-root accumulation pathway, whilst octanol-air partition coefficient and vapour pressure correlated well with air-to-leaf accumulation, and could possibly be used as a prediction of PAH absorbance (Kipopoulou, *et al.*, 1999).

The lipophilic nature of vegetables or plants is still considered the dominant pathway for PAHs accumulation (Kipopoulou, *et al.*, 1999). Therefore vegetables with higher oil content, e.g. carrots or olives are argued to have a higher bioaccumulation of polar pollutants. Olives (20% lipid content) grown for the production of oil near a stockyard for old railway ties, were therefore reasoned to have higher concentrations of PAHs. Corresponding to previous literature, the lower molecular weight PAHs were found in the olive oil at high levels, up to 6.36 ppm (Moret, *et al.*, 2007).

Even though PAHs have been studied more intensively, the volatile fraction of creosote emissions could also potentially taint the waxy layer of plants growing in adjacent areas. Kennison, *et al.*, 2008, proved that compounds from smoke can be absorbed onto the waxy layers of grapes. These compounds 4 ethyl phenol, 4-methyl guaiacol, guaiacol, 4 ethyl

guaiacol and eugenol are not unlike the phenols, e.g. cresol and xylenols that make out the volatile fraction of creosote emissions.

An Australian study conducted on wines affected by bushfires, found that guaiacol, o-cresol, m-cresol and p-cresol were related to the smoky taint found in these wines. These compounds were found at levels 7-36 ppb, 1-52 ppb, 5-11 ppb, 2-6 ppb, 2-9 ppb for guaiacol, phenol, o-cresol, m-cresol, p-cresol and m-cresol, respectively, in commercially affected wines. The study also investigated the glycol-conjugates of these compounds and determined the odour thresholds in red wine. These compounds all contributed to smoky/ashy flavour of these wines (Parker, *et al.*, 2012). These phenolic compounds are present in the volatile fraction of creosote emissions as well.

PAHs do occur in wine at very low concentrations due to the use of toasted barrels during ageing (García-Falcón & Simal-Gándara, 2005). The daily intake of PAHs through wine is calculated at 11-55 ng/person (country and wine specific) and is not considered to be a health risk, as water and food intake as well as breathing (second hand smoke/ pollution) may lead to higher intake of PAHs. The levels found in wine aged in French barrels from two cooperages and wine aged in American oak barrels varied between 0.08 - 0.4 ppb (Chatonnet & Escobessa, 2007). Levels found in Spanish commercial wines were low and ranged from the limit of quantitation (LOQ) to 0.1 ppb, the legal limit in drinking water (García-Falcón & Simal-Gándara, 2005). According to these authors, factors that play a role in the concentration of PAHs present in oak wood are the seasoning of wood, diesel vehicles emissions, the degree of toasting (light to heavy), the temperature of toasting, the method of toasting (open flame vs. convection) and the origin of the wood. Variation in PAHs found between cooperages was ascribed to different wood sources and toasting methods. Higher temperature, the open flame method and higher degree of toasting led to an increase in PAHs (Chatonnet & Escobessa, 2007; García-Falcón & Simal-Gándara, 2005) American wood showed higher concentration of PAHs compared to French oak, as this type of wood needed longer toasting time. The main compounds formed during an increase of intensity of toasting was naphthalene, phenanthrene and chrysene for French oak and naphthalene, acenaphthene, fluoranthene and fluorene for American oak (Chatonnet & Escobessa, 2007).

Many studies have been done on the pollutants, PAHs and VOC, and their fate in the environment and agricultural produce; however few studies have focused on the contamination from creosote as the main source of both PAHs and VOC in environment and produce.

2.5 Analysis of creosote constituents

2.5.1 Sampling and storage conditions

Extensive sampling is often needed in the environment to reflect representative contamination levels of an area (Eriksson, *et al.*, 2001). Glassware is the preferred sampling vessel, as absorption of PAHs may occur with plastic and the glassware should be pre-rinsed to avoid contamination (Hodgeson, *et al.*., 1990). The phenolic compounds emitted from creosote are very volatile compounds, therefore samples analysed for these compounds are should be stored at lower temperatures to decrease their volatility and prevent photolytic decomposition (Hodgeson, *et al.*, 1990). Samples, such as berries and leaves may rot, and are kept below 4˚C to preserve samples from deterioration. PAHs are non-volatile compounds, and due to the double fused benzene ring structure, the compounds are stable, however these compounds are light-sensitive and therefore should be protected from photochemical degradation, e.g. in amber glass or dark room (Hodgeson, *et al.*, 1990). Authors that research creosote contaminated soil samples stored the samples in airtight glass containers at 4˚C, in the dark. The authors noted that at higher temperatures and larger headspace aerobic degradation may occur (Eriksson, *et al.*, 2001). Furthermore if statistical analysis is to be done on the sample results, duplicate or triplicate sampling and analyses is often needed to account for the variability within nature and the analytical system.

2.5.2 Chemical Analysis

Gas Chromatography (GC) and Liquid Chromatography (LC) are most commonly used for environmental and foodstuff analysis. This is due to the compounds of interest's compatibility with these instruments in terms of thermal stability and volatility (GC) and for higher molecular weight compounds their fluorescence and solvent mobility (LC). Many of the preparation methods are the similar for GC and LC, however sample preparation is adapted for the compounds to be analysed and the instrumentation, detection, to be used. Problems faced by researchers are the toxicity of the solvents used for extraction, masking effects of overlapping compounds with the use of chromatography and the preservation of volatile compounds during extractions (Eriksson, *et al.*, 2001).

2.5.2.1 Gas chromatography

Instrumentation and compounds analysed

Gas chromatography (GC) is applied for the analysis of PAHs in environmental samples and is used for identification and quantification purposes. GC is often preferred to liquid chromatography (LC), as it is argued to have better selectivity, resolution and sensitivity

(Poster, *et al.*, 2006). The PAHs have thermal properties, compatible with and GC-MS and can be applied especially for the detection of smaller, more volatile compounds, undetected by LC, e.g. naphthalene and acenaphthene. However GC is only the preferred instrument for the analysis of volatile PAHs, e.g. naphthalene, and other volatile compounds that form part of creosote emissions, such as furans and phenolic compounds. Higher molecular weight PAHs, which are non volatile, sample preparation methods are needed for analysis with GC and LC is therefore often the instrument of choice. Sample preparation methods that are applied for clean-up before analysis with GC/MS includes solid phase extraction (SPE), liquid-liquid extraction (LLE), Soxhlet extraction, solid phase micro-extraction (SPME) (Hale & Aneiro, 1999). In the section below different authors' sample preparation methods in soil, water and foodstuff samples will be discussed.

GC is successfully applied for the analysis of coal-tar associated materials, as these compounds, VOCs and PAHs are often similar in chemical properties (Agency of Toxic substances and Disease Registry (ATSDR), 2003). Mass spectrometry (MS) and flame ionization detection (FID) have been used for the analysis of coal tar (ATSDR, 2003). MS paired with quadrupole electron impact (EI) is suitable for most environmental samples and operated in single ion monitoring (SIM); low detection limits can be achieved. MS is however considered a more sensitive and selective detector and therefore is often preferred for the analysis of complex matrices to FID (Poster, D.L., *et al.*, 2006).

GC was applied for the characterisation of creosote components (WHO, 2004). Compounds were identified using FID, and 63% (w/w) of grade B creosote was determined. GC-FID is also used for the determination of PAHs in environmental samples, e.g. air particulates, water and sediment (Poster, *et al.*, 2006). All residue analysis of creosote is based on the US-Environmental Protection Agency (EPA) method of extraction of PAH, phenols and hetero-cycles (Competent Authority Report, 2007). Analysis of soil, water and air has been developed by various research groups (Eriksson, *et al.*, 2001; Manoli, *et al.*, 2004; Moret, *et al.*, 2007).

Creosote analysis

The volatile fraction of creosote was analysed by GC-MS in a study conducted on newly preserved wood (Gallego, *et al.* 2008), and older wood (Mateus, *et al.*, 2008). For the extraction of the volatile fraction of 20-year-old creosote-treated sleepers, a purge-and-trap method was used. Analytes were recovered with pentane: diethyl ether (1:1) and analysis was carried out by one- dimensional GC, two-dimensional GC (GCxGC), combined with MS detection with quadruple and time-of-flight mass analysers (GC x GC-qMS and GC x GC-ToF-MS) and selective nitrogen-phosphorus detection (GCxGC-NPD). The combination of

these different GC instrumentations allowed for the identification of a complex fraction (Mateus, *et al.*, 2008).

Environmental sample analyses

Various US-EPA methods employ GC/MS for analysis of PAHs in environmental matrices, e.g. volatile PAHs determination in ambient air (Method TO-13A), liquid-solid extraction of organic compounds (Method 525.2, rev. 2.0), semi-volatile organic compound analysis (Method 8270C), amongst others (Poster, *et al.*, 2006). These validated methods are considered standard procedure for analysis of PAH in specified matrices.

A specific method for the extraction PAHs and the degradation products in soil contaminated with creosote was developed using GC-FID (PASHs, PAOHs), HPLC-DAD (basic PANHs, neutral PANHs, neutral metabolites and acidic metabolites) and GC-MS (PAHs) instrumentation (Meyer, *et al.*, 1999). The first step was a Soxhlet extraction with a mixture of dichloromethane and n-hexane. Subsequently the sample was concentrated to 5 ml with a rotary evaporation. SPE cartridges filled with 0.7 g of Chromabond® SB and 2.0 g of silica gel were firstly deactivated with 10% water followed by equilibration with 12ml n-hexane before the extract was passed through. Three fraction were eluted, firstly the PAHs, PASHs and PAOHs with n-hexane/dichloromethane, secondly dichloromethane/methanol for the neutral and basic PAHNs and neutral metabolites. This fraction was further split up by use of cation exchange, as discussed in HPLC sample preparation methods below. Thirdly the acidic metabolites were extracted with methanol and 0.05 N HCl. This method showed good recoveries for most compounds, although not all compounds were satisfactorily extracted. The authors noted that this extraction was time-consuming.

Headspace Solid Phase Micro-Extraction (HS-SPME) and GC-MS is another method described for determination of hydrocarbons in creosote contaminated soil (Eriksson, *et al.*, 2001). Application of this method is less time consuming- as the sample can be analysed directly and the extraction is done on-line, although only the bio-available fraction of contaminants of interest is extracted. A larger sample can be used, giving higher mass of compounds of interest, to be analysed in the concentrated sample. This method was compared to traditional LLE applied with solvents ethyl acetate/hexane (20:80). Soxhlet extraction with toluene, based on a previous study by Hale & Aneiro in 1997, was done to comparatively evaluate the extraction of higher molecular weight PAHs, e.g. < 6-ring aromatic compounds. A large fraction of the compounds of interest in contaminated soil was not extracted with this method. The authors recommended applying this rapid SPE HS-GC method as a screening method for aromatic compounds, 4-ringed compounds and non-polar compounds with high volatility.

Foodstuff analyses

GC-MS is most commonly applied for the determination of organic pollutants in foodstuffs, because of its detection capability of water-soluble lower molecular weight compounds that occurs in plant tissue, e.g. vegetables.

GC was used for the analysis of vegetables irrigated with waste-water. Accelerated solvent extraction (ASE) was used for sample extraction, and clean-up before analysis was facilitated with a silica gel (Tao, *et al.*, 2006; Wang, *et al.*, 2010;). Wang, *et al.*, used selected ion monitoring mode to analyse extracts and the detection limits were 1.5-3.6 ppb dry weight of PAHs. Toa, *et al.*, noted that in the case of the cabbage samples were sulfonated prior to analyses to remove the lipid content.

Smoke derived compounds found in wine due to bushfires were analysed with GC-MS. The compounds found wines made from smoked grapes were guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol, eugenol and furfural (Kennison, *et al.*, 2007). The stable isotope dilution assay analysis was used.

Authors, Chatonnet and Escobessa, 2007, used a SPE GC-mass spectrometry (MS) method optimised for the detection of PAHs in drinking water by Gárcía-Falcón, *et al.*, in 2004, on wine. The wine samples were analysed for PAHs originating primarily from the extraction from the barrels with different levels of toasting. Good results were obtained for the wine samples, even though the method was developed for HPLC, illustrating that the same sample preparation procedures can be applied for GC or LC, if the compounds of interest are the same.

These studies concentrate on the carcinogenic content of the samples and do not investigate sensorial or other quality aspects also affected by the volatile organic compound (VOC)-content from creosote, although some of the volatile constituents of creosote have sensorial properties. Legislative authorities however recommend sensory analysis of food (Agilent application notes, 2010). Very few studies focused on vegetation being influenced by creosote, as most PAH sources were traffic, waste water or bushfires. Further research still needs to investigate the use of biomarkers unique to creosote for environmental samples. 1-Pyrenol is currently used as biomarker in humans to determine the exposure of coke-plant workers to naphthalene and 10 other PAHs (ATSDR, 2003).

2.5.2.2 High performance liquid chromatography

Instrumentation and compounds analysed

HPLC-fluorescence detection (FLD) instruments are commonly used for determination of the higher molecular weight constituents of creosote, e.g. PAHs, and allows for a lower limit of detection (LOD) than GC-FID. High performance liquid chromatography (HPLC) analysis is often applied for the non-destructive separation and identification of coal-derived materials

coupled with ultraviolet (UV) absorbance detection. Furthermore HPLC coupled with diode array UV is becoming increasing common and integrated methods for HPLC and GC/MS are coupled to analyse a larger range of compounds (ATSDR, 2003; Poster, *et al.*, 2006). Other forms of detection can be used to fractionalise molecules in terms of their number of aromatic rings, e.g. ultra violet (UV) or mass spectrometry (MS). This is an accurate method for higher molecular weight molecules, which is not as predominant in environmental samples. Furthermore LC has limited peak capacity making it less suitable for complex mixtures with a variety of pollutants.

Sample preparation techniques that have been developed for HPLC analysis includes Soxhlet extraction, LLE, ultrasonic extraction (UE), accelerated solvent extraction (ASE), microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and solid phase extraction (SPE) and solid phase micro-extraction (SPME) (Wu, *et al.*, 2008). A few PAH analyses methods will be briefly discussed below in agricultural relevant matrices, namely soil, water, vegetables, fruits, wine and olives.

Standard methods are available for the determination of PAH, e.g. EPA 550 for the analysis of PAHs in drinking water. EPA methods are readily available in literature (Poster, *et al.*, 2006). High performance liquid chromatography (HPLC) is the preferred method for higher molecular PAHs that are non-volatile; furthermore PAHs are often fluorescent, making it optimal for HPLC-FLD detection. LC combined with fluorescence detection is an accurate measuring technique of low concentrations of anthracene, pyrelene and benzo[a]pyrene. Benzo[a]pyrene is often used as a target molecule in analysis as it guides as an indication of carcinogenicity (Poster, *et al.*, 2006).

Environmental sample analyses

The US-EPA have developed method 550 for the analysis of PAH contamination in drinking water (Hodgeson, *et al.*, 1990). This method uses liquid-liquid extraction (LLE) sample preparation and high performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence (FLD) detection. The sample preparation procedure is as follows: 1 litre of sample is extracted with methylene chloride (repeat 2 times 60 ml), dried in a column containing 10cm of anhydrous sodium sulphate and concentrated to 1ml. Solvent addition with 3 ml acetonitrile is done before final concentration to 0.5 ml. The big sample volumes and solvent volumes makes this method more expensive and slightly less practical compared to other detection methods discussed here.

A comparative study was done of SPE and SPME sample preparation methods for the detection of PAHs in drinking waters, to determine the method with the best performance in terms of recovery, precision and quantification (García-Falcón, *et al.*, 2004). The SPME research investigated fibre and extract recovery interaction, organic solvent effects, salt

addition, sampling temperature and sampling time. The SPE evaluation included percentage of solvent (acetonitrile addition), storage conditions, organic elution solvent and elution time. SPE was the best sample preparation method and the procedure was setup as follows: SPE C_{18} cartridge was condition with 5ml of acetonitrile, and 10 ml ultrapure water, the sample (250 ml water and 75 ml acetonitrile) was subsequently passed through the cartridge. Washing was done with 20 ml of 30/100 acetonitrile/water followed by 20 minutes of drying under N_2. The PAHs were then eluted with hexane and evaporated to dryness (<60˚C) before re-dissolving in 0.5 ml acetonitrile.

HPLC- diode array detection (DAD) was used for the determination of the neutral and basic polycyclic aromatic nitrogenous heterocycles as well as the respective metabolites in creosote contaminated soil (Meyer, *et al.*, 1999). The advantage of using DAD is that detection can be operated simultaneously at different wavelengths, selective for different compounds. In this study the HPLC was operated at 225, 250, 277 and 281 nm simultaneously to determine the neutral PANHs, whilst 238, 252 and 261 nm were used in the analysis of acidic PANHs. This shows the selectivity of DAD. The authors further noted the importance of analysis of PANHs and their assignment to subclasses as they have different toxicities and biodegradation behaviour and almost similar mass spectra.

QuEChERS extraction has also been used for the determination of PAHs in soil for application to HPLC. QuEChERS was applied as the extraction method in an attempt to decrease the sample preparation time, and to increase recoveries as Soxhlet, LLE and SPE all have evaporation steps that facilitate the loss of volatile compound (Pule, *et al.*, 2010). QuEChERS use acetonitrile, hazardous to the environment, as an extracting solvent, which is compatible for HPLC, which means no evaporation step is needed. Fluorescence detector was set at three different excitation/emission wavelengths and UV detection was used to at 230 nm for the detection of acenaphthylene (Pule, et *al*, 2010). Other authors applied QuEChERS for the determination of PAHs in fish tissue at part per billion (ppb) (Stevens & Szelewski, 2010).

SPE with reverse phase high performance liquid chromatography (RP-HPLC) was used to analyse soil from a creosote contaminated site (Moret, *et al.*, 2007). Soil samples were prepared by liquid-liquid extraction (LLE). One gram of overnight dried soil (40˚C) were pulverized and extracted with 10ml acetone for one hour in an ultra-sonic bath. Thereafter the samples were centrifuged and 5ml of the organic solvent removed using a vacuum evaporator. The remaining 5ml solvent were allowed to evaporate spontaneously at room temperature (usually 25˚C), to prevent loss of the lower molecular weight volatile PAHs. HPLC grade acetonitrile was used to dissolve the residue before analysis.

Foodstuff analyses

A study conducted by Carmargo & Toledo in 2003, measures the human PAHs exposure through dietary intake in fruits and vegetables. The study was conducted on lettuce, tomato, cabbage, apple, grape and pear and measured 10 PAHs, namely fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(e)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene and benzo(g,h,i)perylene.

The vegetables were prepared for HPLC-FLD analysis by boiling a 25g homogenized sample of each vegetable under reflux with 100 ml 2M KOH in methanol for duration of 5 hours. Subsequently the saponified material was separated twice for 2 min in a 500 ml funnel with 150 ml cyclohexane. The organic layer was washed with 100 ml methanol/water (1:1) followed by 100ml of distilled water and concentrated to 50 ml with vacuum rotary evaporation at 40˚C. The cyclo-hexane fraction was extracted three times with N, N-dimethylformamide-water (9:1) and the combined dimethylformamide extract was diluted using 100ml sulphate solution (1%) and further extracted with 50, 35 and 35 ml cyclo-hexane aliquots. The solutions were combined and washed twice with distilled water, dried with anhydrous N_2SO_4 and concentrated to 5 ml with rotary evaporation at 40˚C. A column chromatographic clean up procedure followed where the extract was passed through a glass column packed with 5 g silica gel and anhydrous Ns_2SO_4 and eluted with 85 ml of cyclohexane. The 10-85 ml fraction was collected and dried under a gentle flow of nitrogen to 1ml, and dissolved in 2 ml ACN.

For the determination of PAH in barrel aged alcoholic drinks, including wine, SPE and HPLC was used to determine seven PAHs: benzo[a]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene, benzo[a]anthracene and dibenz[a,h]anthracene. These PAHs are heavy molecular weight molecules, with lower water solubility (García-Falcón & Simal- Gándara, 2005). The SPE extraction method used for concentration and purification of the target carcinogenic PAHs, was optimised using two SPE mini-columns, octadecylsilica and silica stationary phases, in series. Wine and spirit samples alcohol content (v/v) were adjusted to 30-40%, by ethanol addition or dilution. Samples were then loaded onto C18 mini columns under vacuum and rinsed with a 20:80 acetonitrile-water mixture. The columns were dried under nitrogen for 20 min, a silica mini column was attached at the bottom of the C18 column and the PAHs eluted with 10 ml hexane. After detachment of the columns, another 6 ml of hexane was passed through the cartridge. The elute was dried under a gentle stream of nitrogen and re-dissolved in acetonitrile with vortex agitation. After filtering with 0.45 um filters and disposable syringes the samples into amber HPLC vials it was ready for HPLC analysis. This extraction was effective, with slightly lower recoveries for alcohol levels below 30%. This study showed that

the concentration of carcinogenic PAHs found in wine were much lower than concentrations associated with health risks.

SPE with reverse phase high performance liquid chromatography (RP-HPLC) was used to analyse olives near a creosote source (Moret, *et al.*, 2007). The olive samples were also extracted for HPLC analysis using LLE. The sample were ground to a paste before overnight drying at 60˚C, there after a 15 g sample were extracted for an hour in an ultrasound bath using 40 ml of n-hexane. The mixture was then paper filtered before evaporation with a rotary evaporator at 40˚C. The 200 mg of oil aliquot obtained was then further extracted using silica SPE cartridges. This method was developed by Moret & Conté in 2002. Dichloromethane (20 ml) was used for washing and the cartridges were afterwards dried under vacuum before conditioning with 20ml of n-hexane. Elution of the PAHs was done with 8 ml dichloromethane/n-hexane (30:70) mixture. The first 8 ml fraction contained aliphatic hydrocarbons and was therefore discarded. The elute containing the PAH analytes were then concentrated as described above for soil. Acetonitrile, 100 µl, were used to dissolve the residue before analysis.

These studies illustrate that olives, fruits (apple, grape, pear) and vegetables can accumulate PAHs and that various methods has developed for the analysis of PAHs from different sources.

2.6 GENERAL DISCUSSION

Although various environmental studies have been reported in literature, only a few creosote-crop related works have been done (Moret, *et al.*, 2007). Crop studies related to PAHs are usually done on crops grown with waste water irrigation systems or pollution from vehicles and factories or contamination during food processing (Camargo & Toledo, 2003). The sample preparation and analysis of PAHs, VOCs, PANHs and other creosote related compounds have been developed by both GC and HPLC and in some cases standard procedures are in place (Poster, *et al.*, 2006). Alternatives for creosote are recommended by the EU, Australia, Canada and the USA, this movement was done on basis of environmental pollution and the human health risk associated with creosote. In countries where creosote is not as easily phased out, further research opportunities exists regarding the volatile fraction of creosote in relation to the quality of crops grown with these poles as trellis.

2.6.1 Alternatives for creosote

There are various poles available on the market for the use of trellising systems for viticultural practices. Amongst these are metal poles, recycled plastic poles, Copper Chromium Arsenate (CCA) and Copper Chromium Boron (CCB) treated wood poles. The

European (EU) and UK are transitioning from CCA to CCB and in Australia and New Zealand most vineyards use CCA posts (Conradie, 2011). All of these are better options considering the environmental impact of creosote, as they have lower leachate and long life-spans. This is supported by IPW legislation as they have stated: "In view of concerns being raised about the potential negative health and environmental impacts of trellising posts treated with creosote, it may be wise to consider alternatives." Farmers' justification for the prolonged use of creosote poles is due to higher cost of alternatives. No long term studies have been done on the economic implications or the durability of alternatives, although alternative treatments for wood preservation has showed slight concerns for leaching behaviour of chrome, arcane, but Tanalith®, which can be classified as CCB, containing boron, shows a lower environmental impact.

2.6.2 Treatments for polluted sites

There are currently various remediation techniques for creosote contaminated soil, which includes physical, chemical and biological treatments. Of these treatments, biological remediation is a modern and cost-affective approach.

Bioremediation treatments that were carried out on creosote contaminated soil in Kwazulu-Natal, South-Africa, by Atagana, in 2003 included: aeration, adjustment of pH to 7, addition of nutrients, mono ammonium phosphate (MAP) and addition of sewerage sludge in an attempt to increase the microbes in the soil that degrade organic pollutants, e.g. PAHs. The soil was irrigated in dry months to 50-75% field capacity for microbial growth. The experiment showed that aeration and addition of nutrients increase the microbial population in the soil with 40%, whilst a decrease of 27% of creosote contaminants was observed. The addition of sewerage sludge increased the microbial population.

Vermi-remediation refers to the use of earthworms to degrade organic pollutants in soil. A study carried out in Australia by Sinha, *et al.*, in 2008, describes it as a "low-cost and convenient technology" for soil rehabilitation. Species that are capable for accumulating PAHs and pesticides *are Eisenia fetida, Aporrectodea tuberculata, Lumbricus terrestris, Lumbricus rubellus, Dendrobaena rubida, Dendrobaena veneta, Eiseniella tetraedra* and *Allobophora chlorotica.* Earthworms aerate the soil and improve fragmentation of soil, which will increase the microbial population that degrade PAHs. Furthermore they can also bio-accumulate and passively absorb PAHs. Only the 3-4 ringed PAH was found to accumulate in their fatty tissue- probably due to the PAH's lipophilic nature. Earthworms can also digest hydrophobic PAHs by breaking them into aliphatic compounds that can enter the TCA cycle. Bacterial flora species in the intestine of earth worms, e.g. *Pseudomonas, Paenibacillus, Azoarcus, Burkholderia, Spiroplasm, Acaligenes* and *Acidobacterium* help to degrade

hydrocarbons. Fungi species *Pencillium*, *Mucor* and *Aspergillus* also found in the intestine of earthworms degrade hydrocarbons (Sinha, *et al.*, 2008).

A more recent approach studied the possibility of a composting process as a form of mesophilic or thermophilic biodegradation of phenolic compounds in creosote treated wood waste by applying the cellulose degrading actinomycete, *Thermobifida fusca* (Ghaly, *et al.*, 2012). This method would give an effective way of creating a value added product from the creosote wood waste where traditional disposal could lead to the PAH pollution of soil, ground water and surface water. Temperature, pH and moister parameters were used to compare the inoculated process with the control, composting without *Thermobifida fusca*. There was no significant difference found between the treatments and the control. The bioremediation process degraded the phenolic compounds by 73.9% within a 15 day period; a final concentration of 58 ppm material was achieved. Previous bioremediation experiments conducted by McMahon, *et al.*, 2008 only rendered a 66% degradation of the creosote associated compounds. The higher degradation was due to an addition of cooking oil as a source of bio-available carbon, this led to a higher temperature, thus creating better degradation conditions.

2.7 CONCLUSION

Creosote contamination has health implications for foodstuffs, humans and the environment. Areas of contamination should be monitored for the impact on all of the above mentioned factors. Studies conducted on vegetables, olive oils and soil, give insight to the magnitude of contamination. In countries where creosote is not legislated, studies should be conducted on more agricultural crops that could potentially be affected, e.g. grapes grown and wines made from vineyard with creosote trellising poles. Furthermore, the sensorial impact of the odorous fraction of creosote has not been investigated with regard to quality aspects of crops, and further research in this area should be conducted. Chemical analysis methods are available in literature for the determination of environmental samples contaminated with PAHs and thus a basis for these studies already exists. Environmental concerns regarding water, soil and air contamination are important for developing countries and sustainability of their agricultural sector, therefore legislation's recommendation of increased monitoring of contamination should be done. Alternatives and rehabilitation should also be considered in areas that have already been affected.

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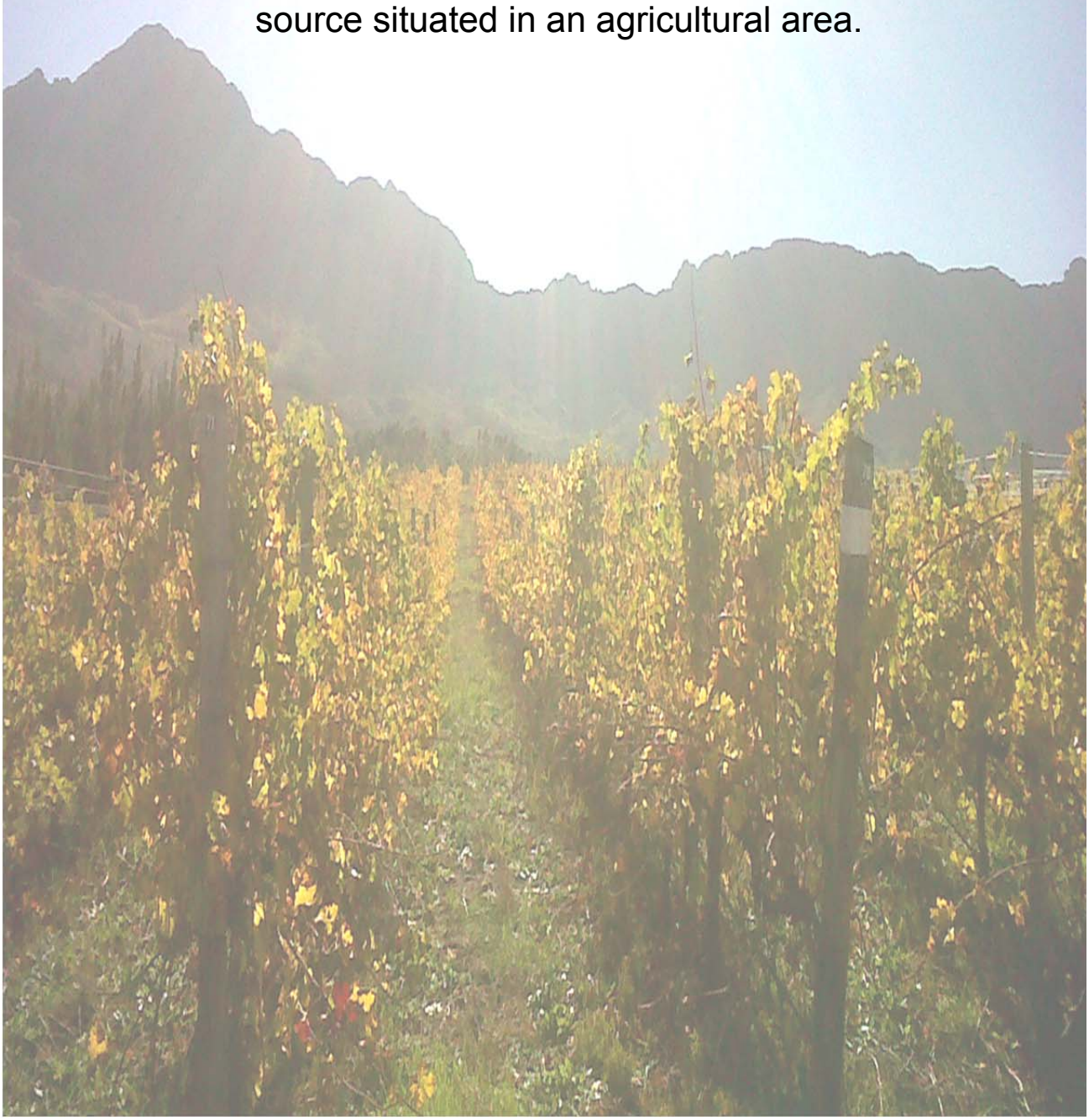
Chapter 3

Research results

The environmental impact on soil and water of an industrial PAH source situated in an agricultural area.



3.1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have been found in various biological matrices. The source of these compounds is frequently industrial activity, in particular the incomplete combustion of organic material (Kipopoulou, *et al.*, 1999). In a natural aquatic environment, authors Brum, *et al.*, (2008), have listed a few possible sources of PAH pollution. These include industrial waste waters, residential run off, petroleum related pollution, groundwater pollution through solid waste area leachate and atmospheric deposition. Crude tar oil, which is used for the distillate product creosote, is also a potential source of PAHs. The active compounds in creosote are made up of an estimated 85% PAHs, thus making a creosote plant a significant environmental risk in terms of PAHs pollution (Agency of Toxic Substances and Disease Registry, 2003). PAHs are not very soluble, especially the higher molecular mass PAHs, and have a higher absorption to particulate matter, binding to the organic content in soils. Low molecular mass PAHs, (<4 ring structures) have been identified in soil as the result of creosote leaching from treated sources (Becker, *et al.*, 2001). Groundwater may also be contaminated with PAHs and serves as a distribution mechanism for these pollutants (Hale & Aneiro, 1997). As a result of leaching, creosote plants have been extensively researched and bio-remediation strategies with microbial degradation processes have been researched by a few groups (Atagana, 2003; Miller, *et al.*, 2004; Sinha, *et al.*, 2008).

The Environmental Protection Act (EPA) has prioritised 16 PAHs, in terms of carcinogenic and toxicity, to monitor the severity of contamination. These compounds are acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene and pyrene. Often benzo(a)pyrene is used as a marker compound, and therefore the concentration limits allowed are occasionally given in terms of a single compound, (benzo(a)pyrene). Various countries have legal limits for PAHs in beverages and foodstuffs (Wenzl, *et al.*, 2007), for example: the US-EPA has a 0.2 parts per billion (ppb or µg/l) limit for benzo(a)pyrene in drinking waters. The Brazilian Health Ministry have set a legal limit of 0.7 ppb for benzo(a)pyrene in drinking waters but the Brazilian Environmental Ministry has set an even lower level in fresh water 0.05 ppb and monitors not only benzo(a)pyrene but also benzo(a)anthracene, chrysene, benzo(k)fluoranthene, benzo(b)fluoranthene, indeno(1,2,3-c,d)pyrene and dibenz(a,h)anthracene (Brum, *et al.*, 2008).

The aim of this study was to determine the environmental impact of a stockyard containing large quantities of freshly creosoted posts on the soil, water and vegetation (leaves) in an adjacent active agricultural area. Representative samples were taken from the area around the stockyard, and PAH analyses were conducted on extracts with high pressure liquid chromatography and diode array detection (HPLC-DAD). The leaves were analysed with

headspace gas chromatography mass spectrometry (HS GC-MS) to determine the volatile organic compounds transmitted from the creosote stockyard into the atmosphere.

3.2 MATERIAL AND METHODS

3.2.1 Sampling and storage of environmental samples

Soil and water samples were taken from the surrounding environmental area of the stockyard to determine the impact of creosote leaching and volatile release. Extensive and intensive sampling is often needed to give a true reflection of an environment's status regarding the exposure to pollutants (Eriksson, *et al.*, 2001). Therefore a representative sample set was taken in triplicate, as outlined below, but only analysed in duplicate, due to time constraints. Soil samples were therefore taken from the stockyard, from areas of plantation, from open field areas, and from the slope down to the river, as some of the compounds are known to spread in groundwater due to their solubility. The rivers were sampled upstream and downstream from the stockyard to determine the quality of the natural waters. Further detail on sampling is included in the next three sections.

3.2.1.1 Soil sampling

The sampling was carried out in late spring and early summer; November, 2011. Soil samples were taken according to guidelines set out by the Cooperative Research Centre of Viticulture (Vitinotes, 2006), which recommended taking the samples at depths where the highest concentration of roots could be found. In this project, vineyards were planted around the stockyard, thus PAHs available to vine root were an important concern. The soil was sampled at eight potentially contaminated plots around the stockyard according to the locations shown in Figure 1, at 0-10 cm, 30 cm and 60 cm depths, to investigate the distribution of contaminants vertically in the soil as well as horizontally over the surface. The sample sites followed the natural slope towards the riverside. PAHs are stable compounds due to the double benzene structure; but can absorb to plastic and are photo-sensitive. Indirect photolysis or photo-oxidation, in the presence of peroxyl, hydroxyl and other radicals seems to be more effective than direct photolysis. Not all PAHs have the same photo-sensitivity and half-lives observed varied from 0.2 hours to 550 days (World Health Organization (WHO), 2004). Therefore samples were taken in 125 ml glass jars and stored at -4˚C to -20˚C in cardboard boxes to protect from the light. Soil was dried before analysis, by exposing it to silica gel in a closed container at a temperature lower than 35˚C, to prevent any volatilisation of compounds of interest.

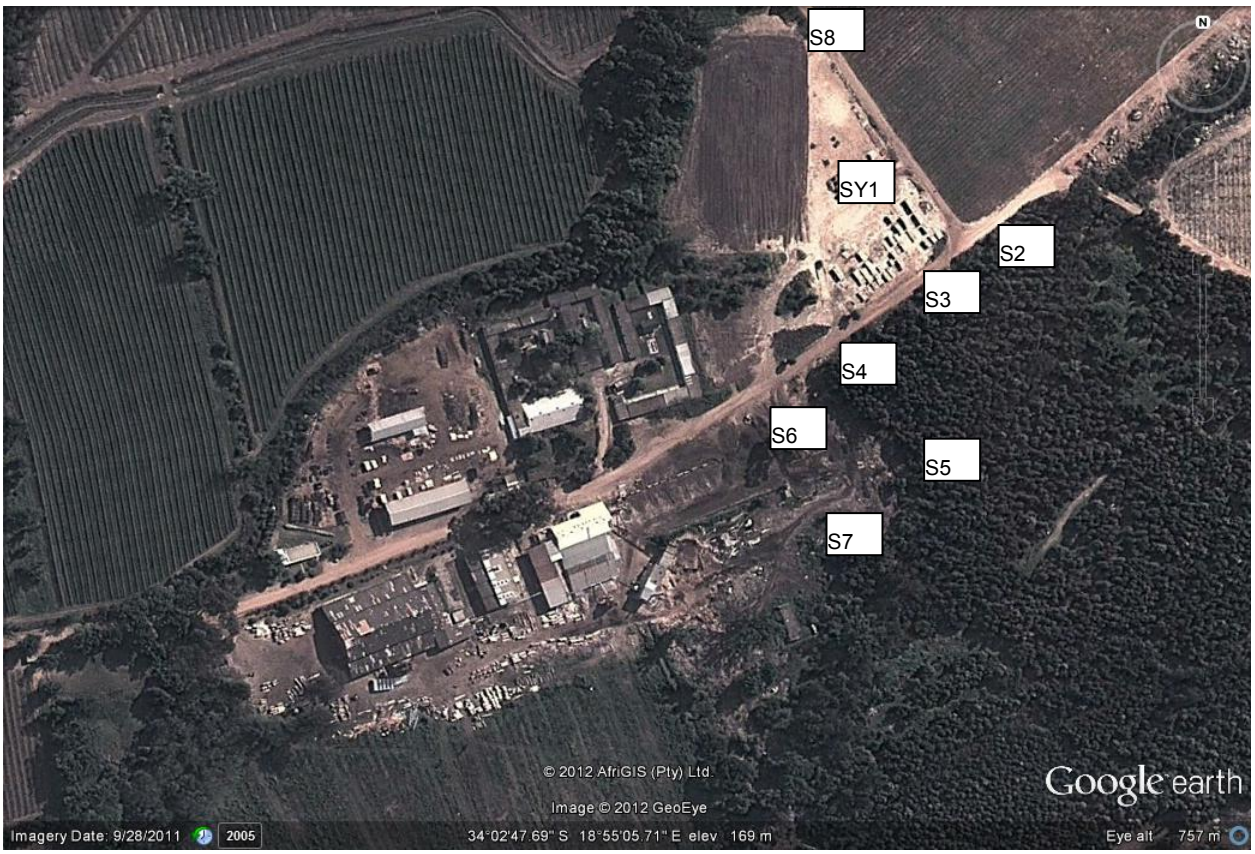


Figure 1: Soil sample locations

3.2.1.2 Water sampling

Concerns of water pollution due to the mobility of PAHs were investigated because a number of streams pass the industrial area surrounding the creosote stockyard. The Lourens River (LR), Amandel River (AR), Lourens Meander (LM) and Fly Fishing (FF) areas were sampled in duplicate to determine the levels of contamination (Figure 2). Sampling of water for the analysis of PAHs was done in coloured glass bottles, 750 ml, with as little headspace as possible, as recommended by Brum, *et al.* (2008). The coloured bottles were used with the aim of preventing photo-degradation of PAHs which could take place during transport or storage (Hale & Aneiro, 1997, Asan & Isildak, 2003). The samples were taken a day before analysis and refrigerated overnight at 4˚C until sample preparation.

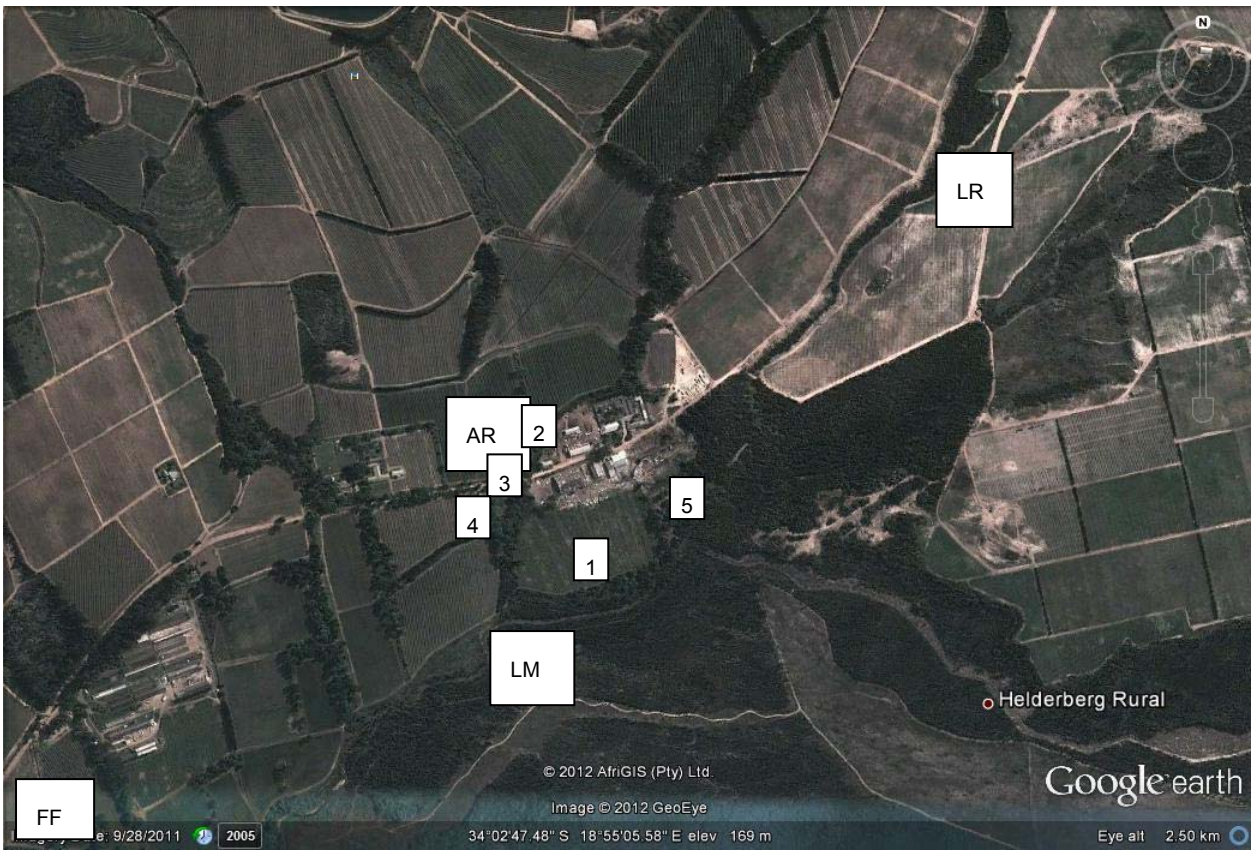


Figure 2: Water and leaves sample locations

3.2.1.3 Leaf sampling

Initially five plots were sampled around the creosoting plot to identify and measure the compounds accumulated in the different vegetation around the stockyard. The leaves sampled include an unidentified broadleaved weed species (plot 1), *Pyrus malus* (apple, plot 4), *Prunus spp.* (plums, plot 2 and 3) and *Eucalyptus cinerea* (Eucalyptus, plot 5) (Figure 2). These leaves all differed in morphology and texture, and were sampled from different heights. Due to time constraints, only a single sample was analysed and thus no statistically analysis were done, only estimated concentrations will be given in the results section.

3.2.2 Analysis of PAHs

3.2.2.1 Reagents

All chemicals used for sample preparation were liquid chromatographic grade: acetonitrile (Sigma-Aldrich and LiChrosolv®), n- hexane (Sigma-Aldrich) and acetone (Sigma-Aldrich). The ultrapure water was purified with a MilliQ system (Millipore, Bedford, Mass., USA). Pure compounds of the priority PAHs for this study were Reagent grade and were purchased as follows: benzo(a)anthracene, chrysene and anthracene from Fluka Analytical (Steinheim, Germany) and benzo(a)pyrene, acenaphthene, acenaphthylene, fluoranthene, naphthalene, phenanthrene, pyrene and fluorene from Sigma Aldrich (Steinheim, Germany). Stock solutions of 1000 ppm were made up in acetonitrile for analytical work. These solutions were kept

refrigerated below 4˚C, and fresh stock was made before analysis. A mixture made from the individual 16 priority EPA PAHs were made up in 1000 ppm stock solutions. The stock solutions were used for spiking, validation and recovery studies as well as the calibration levels, for soil and water procedures. The internal standard, 2-ethyl anthracene (Sigma Aldrich) was made in stock solution (2.5 ppm). Data analysis and integrations was carried out with Chemstation® (Agilent) and Statistica® software (Statistica 10.0, Statsoft, USA)

3.2.2.2 Calibration

A seven point calibration was set up for the PAHs measured in soil, these included benzo(a)anthracene, chrysene, anthracene, benzo(a)pyrene, acenaphthene, acenaphthylene, fluoranthene, naphthalene, phenanthrene, pyrene and fluorene. Silica (filter) sand, rinsed with distilled water, was spiked at 25, 50, 100, 250, 500, 1000, 1500 parts per trillion (ppt or ng/l) and extracted with soil liquid- solid extraction (LSE) sample preparation method adapted from EPA method 3550 C, as described in sample preparation section. Method development was carried out as described in sample preparation section. The method validation and a recovery study were also done prior to analysis, but forms part of another project within the study group.

For the few water samples to be analysed, two reference concentrations was prepared in triplicate to quantitate concentrations found in the samples. The method used for sample preparation was validated and developed by García-Falcón *et al.*, in 2004. Reference standards were prepared by spiking ultrapure water with the PAHs, as described in soil, and extracted with the solid phase extraction method (SPME). The reference points were prepared and analysed in triplicate and were at 0.3 and 30 parts per billion (ppb).

Table 1: HPLC-DAD calibrations and R^2 for environmental samples (soil and water)

HPLC-DAD	Soil calibration		Water reference	
Environmental PAHs	**Calibration equation**	**R^2**	**Reference equation**	**R^2**
Fluorene	y= 0.0 x + 0.004	0.99	n.d at lower level	
Phenanthrene	y= 0.0 x + 0.013	0.999	y = 0.506 x + 0.043	1
Anthracene	y= 0.001 x + 0.003	0.996	y = 1.111 x + 0.476	1
Benzo(b)fluoranthene	y= 0.0 x - 0.0	0.999	y = 0.474 x + 0.045	1
Benzo(a)pyrene	y= 0.001 x - 0.005	0.999	y = 0. 727 x + 0.001	1
Chrysene	y = 0.001 x - 0.019	0.993	y = 1.113 x - 0.049	1
Benzo(a)anthracene	y = 0.000 x + 0.014	0.992	y= 0.586 x + 0.009	1
Acenaphthylene	y = 0.0 x - 0.028	0.938	n.d in water	
Acenaphthene	y = 0.000 x - 0.029	0.968	n.d in water	
Fluoranthene	y = 0.0 x + 0.015	0.998	y = 0.191 x + 0.017	1
Pyrene	y = 0.0 x + 0.01	0.992	y = 0.604 x + 0.035	1

Not all the compounds had satisfactory correlation coefficients, therefore only compounds with $R^2 > 0.99$ were used for quantitation.

3.2.2.3 Sample preparation

Sample preparation had to be adapted for the various biological matrices, as each matrix and method has its own interferences and limitation. The sample preparations for soil and water were adapted for HPLC-DAD from existing methods in literature, and will be described below.

Liquid Solid Extraction of Soil

A liquid solid extraction (LSE) procedure adapted from the EPA 3550C method for PAHs in soil was used for analysis of soil samples. This method was adapted as follows: 2.5 grams of dried, homogenised soil was extracted with 5 ml of n-hexane/acetone (4:1) solvent mixture using ultra-sonication for 3 minutes, three times over. The internal standard used, 2-ethyl-anthracene (2 EA) was added with the solvent to the soil, 0.5 ml of 2.5 ppm, during the first extraction step. The aliquots were separated from the soil after each sonication and added together, thus the final volume of the aliquot was ±15 ml. The extraction was centrifuged at 4000 rpm for 4 minutes, to ensure a clean extraction without excess soil particles. The top layer of solvent containing PAHs is then separated and evaporated to dryness under a gentle stream of N_2 at 40˚C in a sample concentrator (Techne DB-3 series Driblock (UK)). The PAHs, which do not volatilise at 40˚C, adsorbs to glassware. The compounds were then dissolved in 1ml acetonitrile, agitated by vortex and finally passed through 0.45 µm nylon filters (Agela Technology) with a 1 ml syringe (Surgi-plus) into a brown HPLC grade vile for analysis on the Agilent HPLC-DAD system. Various other sample preparation methods have been developed in literature; these include solid-phase extractions (SPE) and Soxhlet extraction.

Authors Pule, *et al.*. developed a QuEChERS method in 2010 which excludes an evaporation step. The authors stated that the evaporation step may lead to lower recoveries due to evaporation of the volatile PAHs; therefore this method was compared to the EPA 3550C LSE method to determine the most efficient method for final analysis. The QuEChERS extraction procedure used a 5 g soil sample, previously dried and sieved, in the SampliQ QuEChERS centrifuge tube included in the extraction kit (Agilent Technologies). Samples were spiked with the compounds of interest (16 EPA PAHs) from the stock solution made up in acetonitrile and the internal standard (2 EA); the samples were spiked at 300 ppt. Thereafter 5 ml of water was added and the tube was vigorously hand shaken for 1 minute. Acetonitrile (10 ml), 6 g anhydrous magnesium sulphate ($MgSO_4$) and 1.5 g of anhydrous sodium acetate (NaOAc) (Agilent Technologies), included in the extraction kit, was added subsequently. The samples were then vigorously hand shaken for 1 minute followed by centrifugation at 4000 rpm for 5 min. The SPE- cleanup procedure used the 6 ml aliquot from the top organic layer and transferred this volume to the SPE 15 ml tube, also included in the kit, containing 400 mg of primary and secondary amine exchange material (PSA), 400 mg of C18EC and 1200 mg of anhydrous $MgSO_4$ (Agilent Technologies). The samples were again vigorously hand shaken and centrifuged at 400rpm for 5min before the final 4 ml aliquot was filtered using a 0.45 µm

nylon filter (Agela Technologies) and a disposable syringe (Surgi-plus). The 1 ml extract was placed in an amber HPLC grade vial and kept refrigerated until analysis.

Solid Phase Extraction of Water

Water was collected from the potentially contaminated natural waters surrounding the creosote stockyard as well as further downstream of a nearby river. The water samples were analysed using an adapted method developed by García-Falcón *et al.* in 2004. These authors investigated various factors that contribute to the recovery and sensitivity of sample preparation method. The samples was then analysed on a HPLC-FLD instrument. They reported detection at 2-30 ppt. We adapted this method by means of testing various C18 cartridges and with an increased spiking level for analysis on HPLC-DAD to determine the limit of detection (LOD). Due to the limited samples that were sampled (duplicates of 4 water samples), no validation was done on this method and only a two point reference calibration was set up. The procedure was further followed as described in García-Falcón *et al.*, 2004.

The Bond Elut C18-OH cartridges (1 gram, 6ml) from Agilent Technologies were conditioned using 5 ml acetonitrile followed by 10ml of ultrapure water. The sample, 250 ml of water, with a 75 ml of acetonitrile addition was then passed through the cartridge. The cartridges and glass bottles used for the samples were washed with 20 ml of the same ratio of water to acetonitrile (100/30), using ultrapure water in this step. After washing the cartridges were dried under vacuum for 20 min. Thereafter the PAHs were eluted using 5 ml of n-hexane and collected in clean glass vials. The collected elute were evaporated under a gentle stream of N_2 in a sample concentrator (Techne DB-3 series, Driblock (UK)) from the samples were heated to 40˚C during evaporation, which aids in evaporation of the solvent without volatilization of the PAHs. The compounds were finally dissolved in 0.5 ml acetonitrile by means of vortex agitation. The final sample was filtered through 0.45 µm sterile nylon filters attached to 1 ml syringes (Agela technologies and Surgi-plus) and placed in brown labelled vials at 4˚C until analysis. The samples in amber flasks would be stable for a six month period (García-Falcón, *et al.*, 2004), but all samples were analysis within a two day period.

During trial preparations 250 ml of ultrapure water was spiked with the 16 EPA PAHs and the internal standard (2 ethyl anthracene) at 30 and 300 ppt. The lower concentration, 30 ppt, was not detected, probably due to the lower sensitivity of DAD detection compared to FLD used in García-Falcón, *et al.*, study.

Leaves sample preparation

Leaves were weighed to five grams and cut up into small pieces, before homogenization with an IKA® T-18 Basic Ultra Turrax homogenizer. Homogenization was not possible without the addition of 10 ml methanol:water (30/100) solvent. This solvent was chosen for compatibility with Headspace GC-MS analysis. Five grams of this methanol:water leaf paste was weighed

out in a GC-MS grade vial containing 1 g of NaCl. The addition of salt to a sample increases the ionic strength of the solution to aid in the efficiency of the extraction (Wu, *et al.*, 2008), which was done online using a SPME 65 µm polydimethylsilxane/divinylbenzene (PDMS/DVB) fibre (Sigma- Aldrich). Online agitation of samples was carried out at 250 rpm and extraction took place at 50˚C for 20 minutes, thereafter the compounds were desorbed into the GC injector for 1 minute.

3.2.4.2 Instrumentation

High pressure liquid chromatography

Analysis for PAHs in environmental samples, soil and water, were done using an Agilent 1260 Infinity HPLC-DAD instrument from Agilent Technologies Inc. (Palo Alto, CA). The system was equipped with a C18 reverse-phase column (Poroshell® 120), with diameters 4.6 × 50 mm and i.d. 2.7 micron particle size (Agilent). The column was maintained at 25˚C during analysis. The mobile phases were ultrapure water (A) and acetonitrile (B), and a flow rate of 1 ml/minute was maintained. The chromatographic conditions were programmed as follows: 0-2 minute of 40% B, 2-22 minutes of 40-80% B, 22-26 minutes of 80-100% B and 26-29 minutes of 100% B. The total run time, including reconditioning, was 34.5 minutes. The Diode Array Detector (DAD) was programmed to monitor readings form 190-400 nm for the identification of PAHs. Quantification was done at 230 254 and 270 nm. Chemstation® software from Agilent (Palo Alto, CA) was used for identification and data analysis.

Headspace gas chromatography

Agilent technologies network GC system, model 6890N, coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD), model 5975B, (Agilent Technologies Inc., Palo Alto, CA) was used for all separations of the volatile compounds. A FFAP column (60m × 250 µm × 0.50µm), model J&W 122-3263, from Agilent Technologies was installed for the analysis of volatile phenols. Helium gas was used as the carrier gas, at a flow rate of 1ml.min^{-1}. The injector was operated in splitless mode and maintained at 240˚C. The oven programme was operated at an initial temperature of 40˚C for 3min, thereafter a ramp to 100˚C for 2min, second ramp to 180˚C for 5min and the final ramp to 240˚C for 15min. The MSD was operated in Scan and SIM mode to obtain the data; for quantification, only SIM mode was used, scan parameters were 15 for low mass and 300 for high mass. The MS source and the quad were maintained at 240˚C and 150˚C, respectively. Compounds were identified using GC-MS retention times and identification of the mass spectra with NIST05 spectral library collection. The total run time of the method was 44 minutes.

3.3 RESULTS AND DISCUSSION

The results obtained were part of a bigger project where vineyard leaves, berries and wines were investigated for volatile organic compounds (VOC) by GC-MS and polycyclic aromatic hydrocarbons (PAHs) with HPLC-DAD (Chapter 4).

3.3.1 Soil analytical data

During method testing the adapted liquid-solid extraction (LSE) method was compared to a QuEChERS method developed by Pule, *et al.* in 2010.

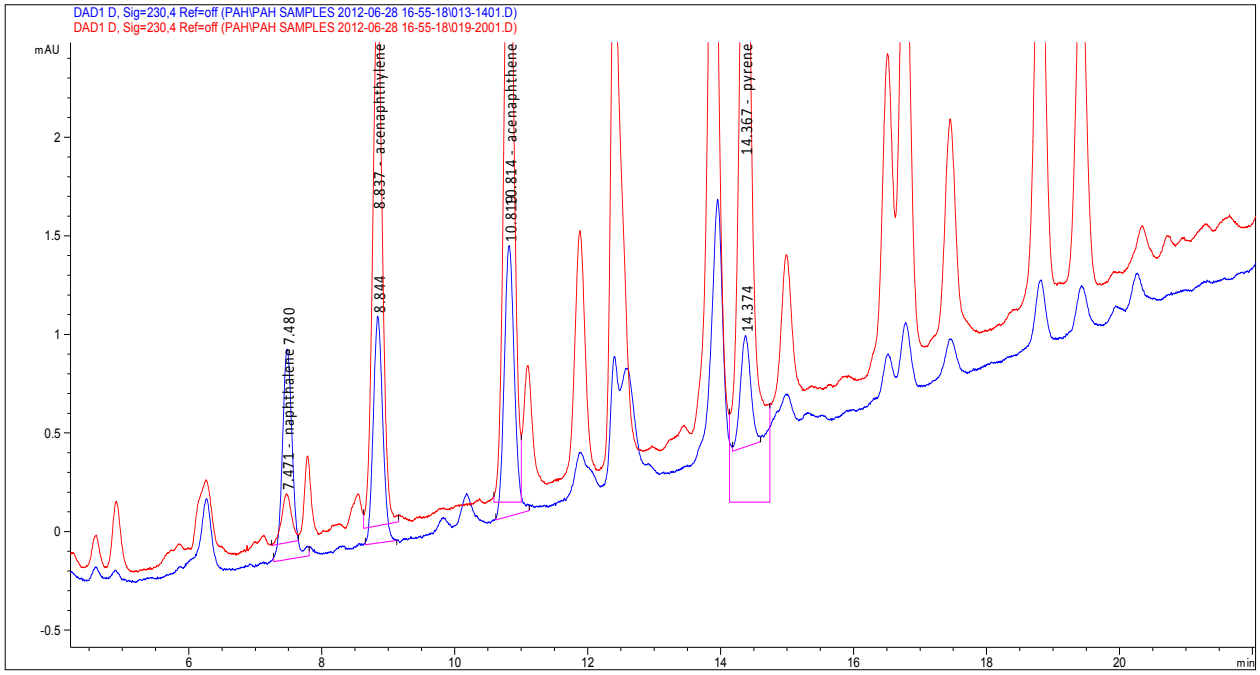


Figure 3: Comparison of LLE and QuEChERS methods. Red chromatograph=adapted LSE method and blue chromatograph = QuEChERS (Pule, et al, 2010).

The two methods both showed the separation and identification of the compounds, the LSE method (red chromatograph) did however show a better signal to noise ratio, as indicated by the larger red peaks (Figure 3). Other factors tested were technical repeats, extracted reproducibility and integrated peak correspondence with identified peaks and compounds, which was satisfactory for both these methods. The consistency of the LSE procedure was very good, indicating the effective retention of the PAHs at a 40˚C during the evaporation step. This showed that the potential loss of compounds of interest through volatilization during the vaporization step, as indicated by Pule, *et al.*, was not an issue in this case. This agrees with results found by Wu, *et al.*, in 2008 where naphthalene, phenanthrene and anthracene decreased during sample preparation only when temperatures above 40˚C were applied. Fluoranthene and pyrene however were stable at temperature up to 60˚C.

The adapted EPA method was applied for all soil samples collected from the various plots around the stockyard. Calibration, recovery studies and method validation for various soil types were done before routine analysis was performed.

3.3.2 Soil sample results

The PAHs detected in soil was phenanthrene, benzo(b)fluoranthene, benzo(a)pyrene, chrysene, benzo(a)anthracene, pyrene, dibenz(a,h)anthracene and benzo(g,h,i)perylene. The concentrations varied from the site and the depth of the samples. The highest concentrations found in the majority of the samples were from site 1, 4, 6 and 7 and 10 cm depth (Figure 4 and 5). Benzo(a)pyrene is used as a marker molecule for PAH contamination and thus the ANOVA results for benzo(a)pyrene is given in Figure 4 and 5.

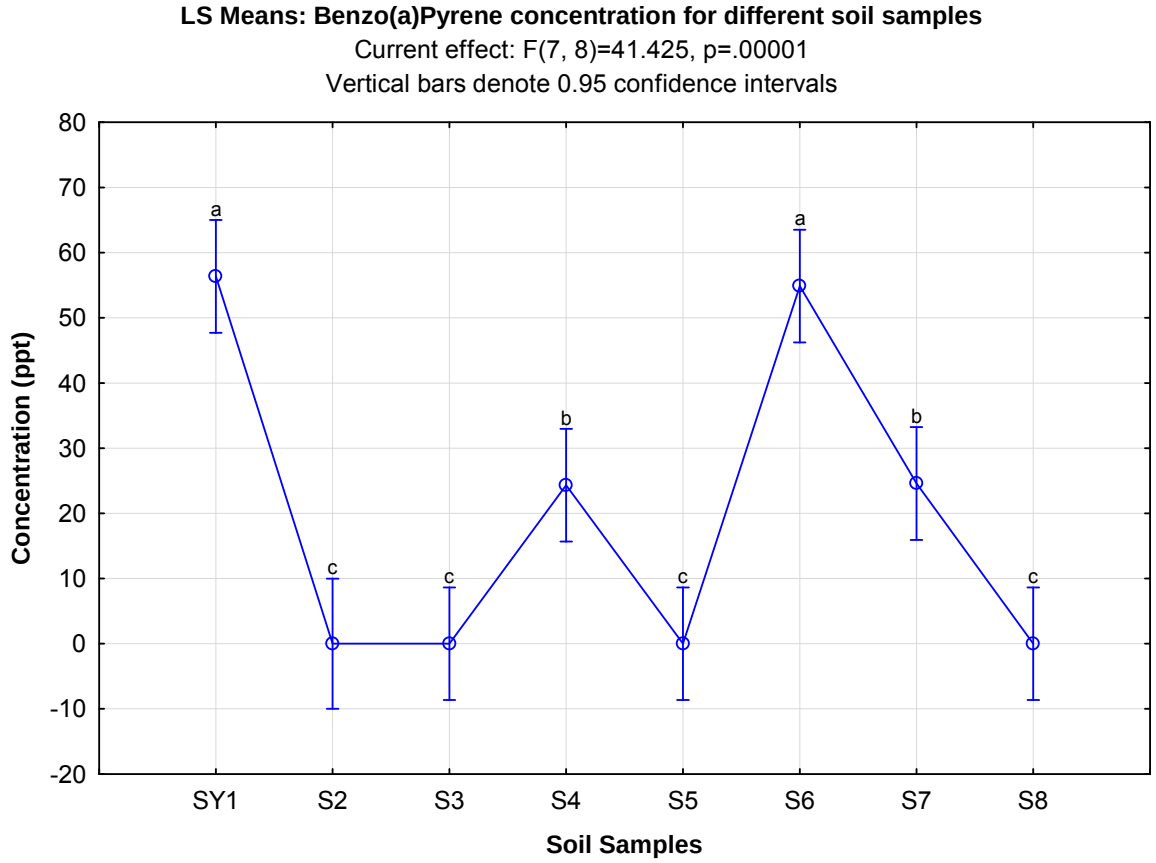


Figure 4: The ANOVA least squares plot showing the concentration of Benzo(a)Pyrene found in highest in soil samples from site 1,4,6 and 7 from the 8 different sites surrounding the stockyard.

The concentrations of benzo(a)pyrene found varied from the different localities where the samples were taken. The highest concentration found were from the stockyard and from sample site 6 (Figure 4), which is located in the industrial area across from the stockyard (Figure 1). Site 4 and 7 had significantly lower concentrations of benzo(a)pyrene present, but significantly higher than site 2, 3 and 5 where no benzo(a)pyrene was found (Figure 4). This may indicate that the whole area from site 4, 6 and 7 could be contaminated with PAHs from creosote related activities. All the PAHs, with the exception of chrysene, had the highest

concentration in the top layer of soil taken at 10 cm depth. Benzo(a)pyrene, benzo(a)anthracene, benzo(g,h,i)perylene and phenanthrene showed a decrease in concentration with the increase in depth of soil. This may indicate that these compounds are restricted to the top layer of soil. Benzo(b)fluoranthene, pyrene and dibenz(a,h)anthracene had highest concentration in top soil but was prominent in 60 cm depth; indicating the ubiquitous nature of these specific compounds. The only compound found in highest concentrations at deeper levels was chrysene which was found in 60 cm. Pyrene was present in all the samples except the sample taken in the vineyard.

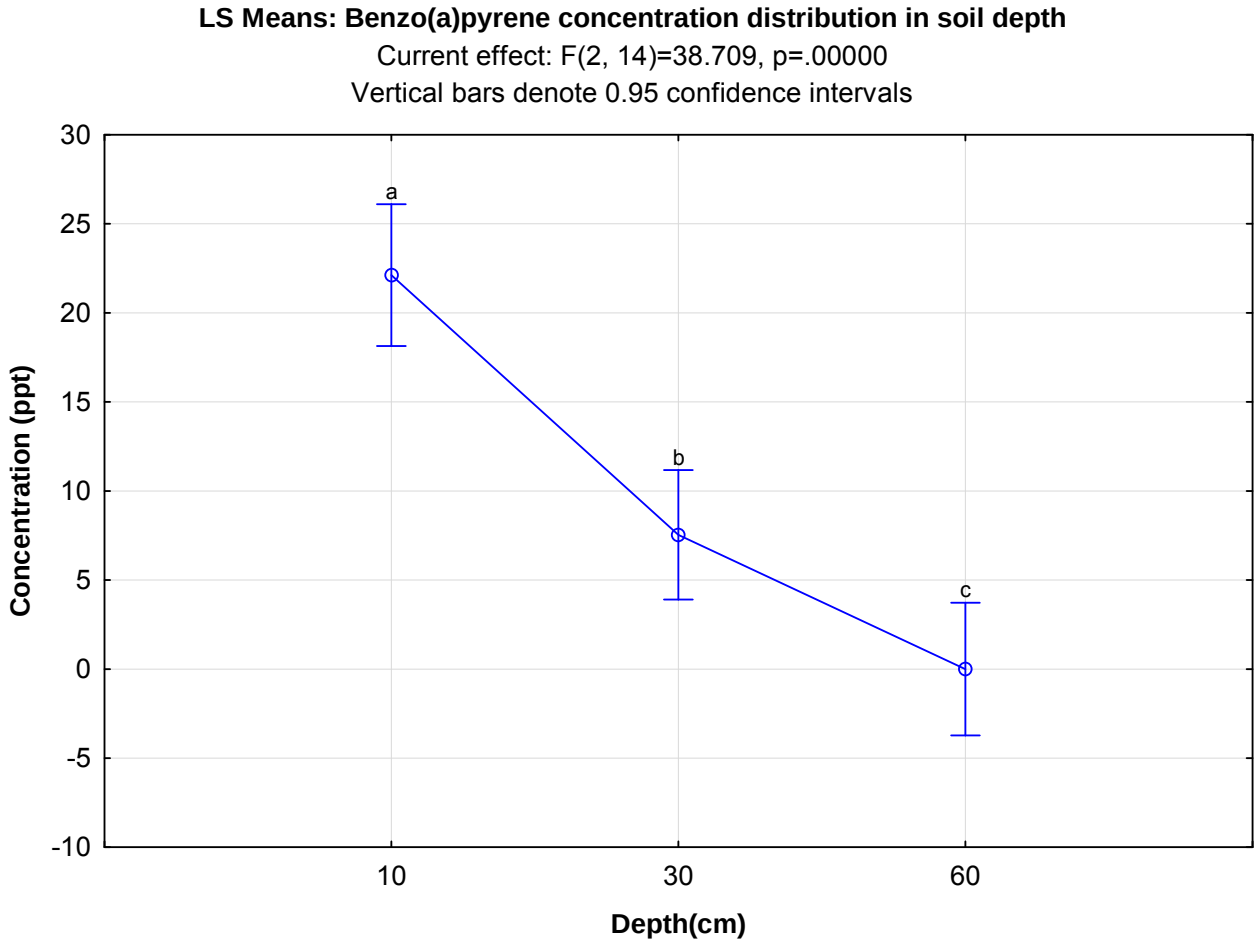


Figure 5: ANOVA least square means plot indication the decrease in benzo(a)pyrene (ppt) distribution in soil according to sample depth**.**

Table 2: Mean and standard deviation of PAHs found in soil samples

Environmental samples	Phenanthrene	Benzo(b) fluoranthene	Benzo(a)pyrene	Chrysene	Benzo(a) anthracene	Pyrene	Dibenz(a,h) anthracene	Benzo(g,h,i) perylene	Total PAHs
Soil	*Mean ± σ*	*Mean ± σ*	*Mean ± σ*	*Mean ± σ*	*Mean ± σ*	*Mean ± σ*	*Mean ± σ*	*Mean ± σ*	*∑ PAHs*
Stock yard	1.37 ± 2.75	187.2 ± 77.67	56.35 ± 10.54	19.85 ± 14.21	n.d	24.94 ± 28.79	28.44 ± 19.13	61.42± 15.49	379.58
S2	n.d	n.d	n.d	7.93 ± 15.87	n.d	41.89 ± 28.22	n.d	n.d	49.83
S3	n.d	n.d	n.d	n.d	n.d	11.42 ± 22.84	n.d	n.d	11.42
S4	n.d	36.2 ± 72.42	24.33 ± 28.1	17.28± 20.01	n.d	22.7 ± 26.24	20.11 ± 23.6	n.d	120.65
S5	n.d	n.d	n.d	n.d	n.d	35.99 ± 71.98	n.d	n.d	35.99
S6	n.d	44.15 ± 88.3	54.86 ± 17.32	34.09 ± 12.88	17.76 ± 35.52	103.13 ± 37.76	n.d	n.d	253.99
S7	5.1 ± 5.88	73.92 ± 85.37	24.58 ± 28.4	26.11 ± 5.88	22.5 ± 26.06	86.8± 31.74	n.d	n.d	239.00
S8	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.00

The total PAHs found, was highest for the soil sample taken from the directly from the stockyard, 379.58 parts per trillion (ppt) which is 0.38 ppb (Table 2). The other sample locations had lower concentrations than the stockyard, as was expected. Sites 6 and 7 (S6 and S7) across from the stockyard, had high concentrations compared to the rest, 253.99 and 239 ppt, respectively. The concentrations reported in creosote contaminated soil by other authors were much higher than was found in this study. Moret, *et al.*, reported from a study conducted in 2007 that the highest concentrations of PAHs found in soil close to a creosote fence was 2157 ppb (fluorene, phenanthrene, anthracene, fluoranthene and pyrene) and 3122 ppb for benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene.

These concentrations were more than a thousand times higher than the concentrations found in this study. Moret, *et al.*, did however investigate closer proximities to the source of contamination and noted a rapid decrease in concentration as the distance increased, therefore the samples taken from 3-10 meters from the stockyard may show these lower concentrations due to the distance from the source of pollution. The summer temperatures experienced is also very high in South Africa, thus some of the more volatile compounds, e.g. naphthalene and acenaphthene, could have evaporated from the stockyard and surrounding polluted soil.

3.3.3 Water analytical data

The SPE water preparation method, adapted from the García-Falcón method, was firstly tested using various C18-cartridges at 30 ppt and 300 ppt.

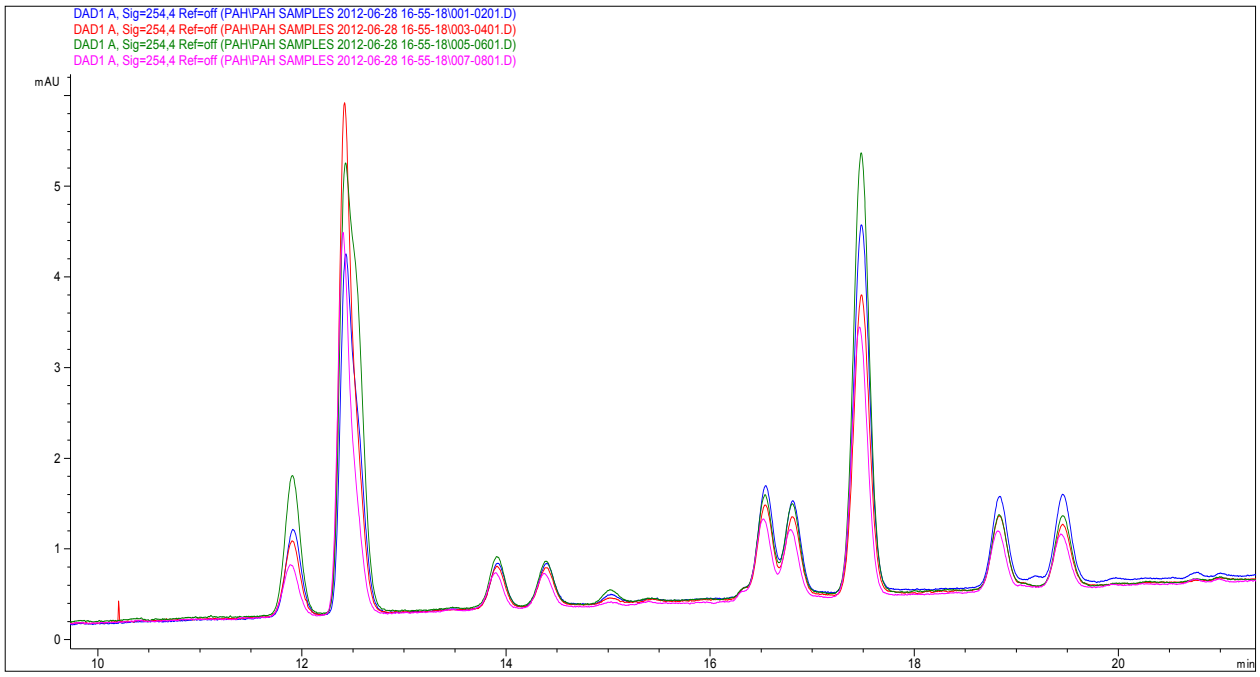


Figure 6: The chromatograph compares the four C18-cartridges tested during method testing. The PAHs were spiked at 300ng/l. The blue chromatograph = Chromabond, red= Varian HF C18-OH, green=Discovery (DSc) 18 LT, 3ml and pink=Discovery (DSc) 18, 3ml.

From the HPLC-DAD data obtained no significant differences were observed between the different cartridges (Figure 6). Therefore the cartridge used for analysis of samples was decided on the basis of being user-friendly and cost effective. Bond Elut C18 (the equivalent of Varian HF from Agilent Technologies) cartridges were used for the samples and reference standards.

3.3.4 Water samples results

The samples taken were from the Amandel River (AR), Lourens River (LR), Lourens Meander (LM) and Fly Fishing pond (FF) (Figure 2) and analysed in duplicate. The water samples analysed only had concentrations of phenanthrene and chrysene present. The total PAH concentrations found in water ranged from 0.085 ± 0.054 to 0.422 ± 0.129 ppb for Lourens Meander and Amandel River, respectively.

Table 3: Mean standard deviation, standard error and confidence interval for Phenanthrene concentrations in river water samples.

Descriptive Statistics Phenanthrene							
	Samples	*n*	*Mean*	*σ*	*Standard Error*	*-95%*	*+95%*
Total		**8**	**0.10266**	**0.05267**	**0.01862**	**0.05863**	**0.14669**
Sample	**AR**	**2**	**0.10167**	**0.01568**	**0.01109**	**-0.03918**	**0.24253**
Sample	**LM**	**2**	**0.08547**	**0.03600**	**0.02546**	**-0.23799**	**0.40893**
Sample	**LR**	**2**	**0.15433**	**0.09435**	**0.06671**	**-0.69336**	**1.00202**
Sample	**FF**	**2**	**0.06916**	**0.02824**	**0.01997**	**-0.18456**	**0.32287**

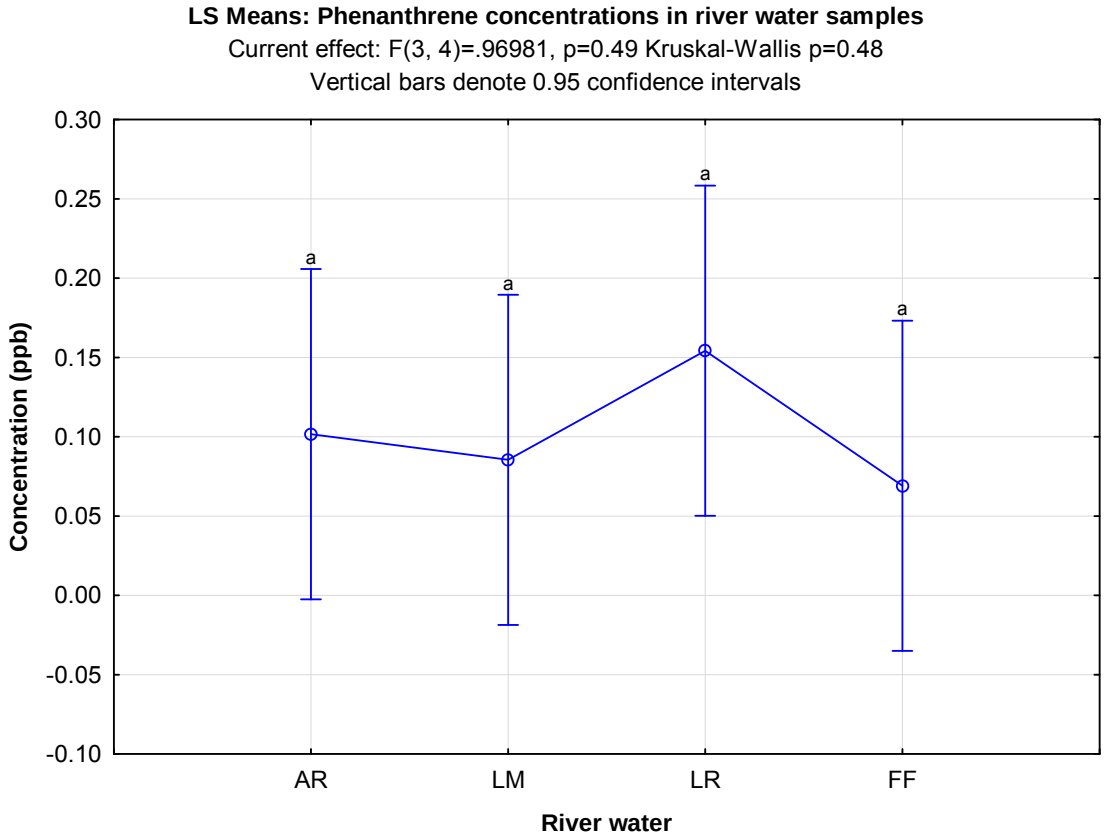


Figure 7: The ANOVA least square means plot for phenanthrene concentrations in different water samples shows no significant differences between samples.

Table 4: Mean, standard deviation, standard error and confidence intervals for Chrysene concentrations in river water samples.

Descriptive Statistics Chrysene							
	Samples	*n*	*Mean*	*σ*	*Standard Error*	*-95%*	*+95%*
Total		**8**	**0.15491**	**0.16673**	**0.05895**	**0.01553**	**0.29430**
Sample	**AR**	**2**	**0.32015**	**0.03956**	**0.02797**	**-0.03530**	**0.67560**
Sample	**LM**	**2**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
Sample	**LR**	**2**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
Sample	**FF**	**2**	**0.29951**	**0.02459**	**0.01739**	**0.07858**	**0.52043**

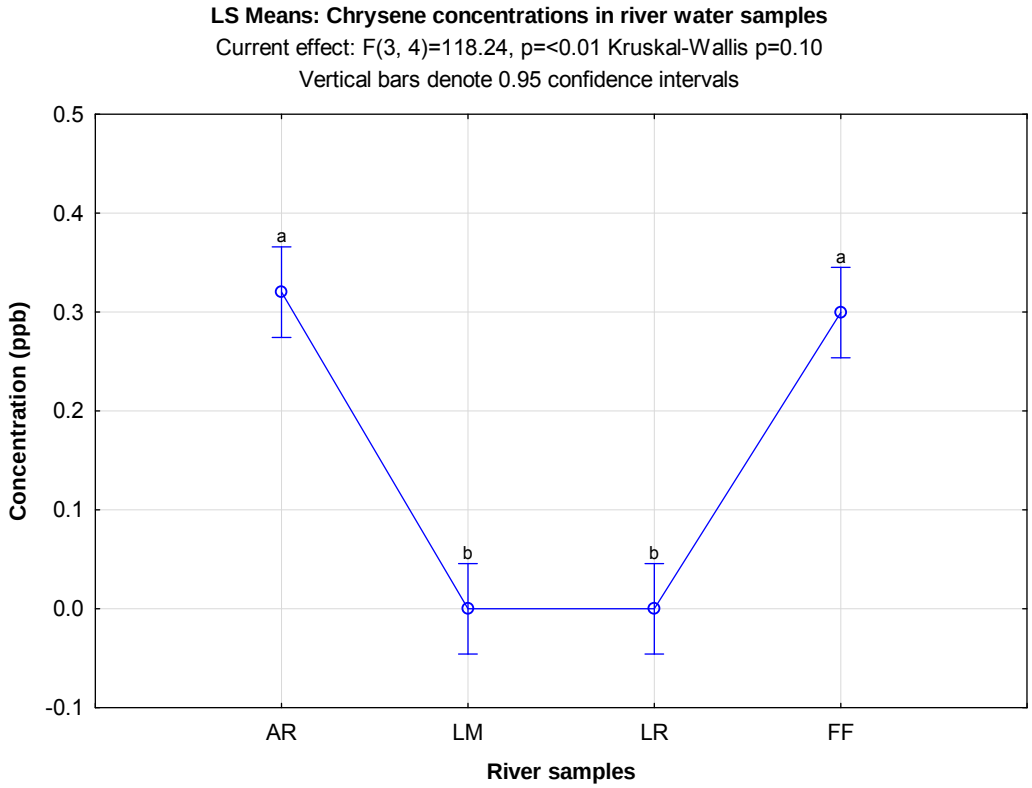


Figure 8: The ANOVA least square means plot for chrysene concentrations (ppb) for different water samples.

There were no significant differences between phenanthrene concentrations found in the samples (Figure 7), but the Lourens River sample had the highest concentration of phenanthrene (Table 3). Only two of the samples had chrysene present and these concentrations were significantly higher than the samples without chrysene (Figure 8). The samples with chrysene concentrations were the Amandel River (closest to the stockyard) and the Fly fishing stream where water streams unite in dams and an accumulative effect is possible. The water samples free of chrysene were from the Lourens River, which is sampled upstream from the stockyard, thus before contamination could take place and Lourens Meander, which is where the Lourens and Amandel Rivers join. The contribution of the Lourens River would dominate, and thus contamination in the Amandel River could be diluted. The concentration of chrysene found ranged from -0.03530 to 0.67560 ppb for the Amandel River and 0.07858- 0.52043 ppb for the flyfishing stream (Table 4). The concentrations found for chrysene was much higher than measured for phenanthrene. A study conducted on river water contaminated by creosote in the USA (WHO, 2004) reported concentrations of anthracene (400-38 700 ppb), benzofluoranthenes (n.d. -5500 ppb), benzo(a)pyrene (300-6600 ppb), fluoranthene (1200-110 000 ppb), fluorene (600- 12 300 ppb), naphthalene (700-14100 ppb), phenanthrene (2300- 155 00 ppb), pyrene (2100-85 000 ppb). The authors did not detect chrysene concentrations and the phenanthrene concentrations were much higher than the values reported here.

This could be due to the high water rainfall in the winter season of 2012, and the fact that only surface river water was analysed and not sediment. Another factor that could contribute to the lower concentrations found in this study is that phenolic compounds were not included. It has been reported that only the lower molecular weight PAHs and phenolic compounds are soluble in water, and these compounds would thus be more likely to be found in contaminated water.

The use of raw water is often used in the agricultural, food and beverages, industries and is recommended to meet drinking water standards (García-Falcón, *et al.*, 2004). South African drinking water standards were investigated by Mamba, *et al.*, in 2008 and found no legal limit for the sum of PAHs in South Africa, whilst the Netherlands and European Union had legal limits of 0.1 ppb for benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene. Furthermore Directive 98/83/EU specifies that the concentration of benzo(a)pyrene should be 10 ppt.

None of these compounds were found in this study, but the total for chrysene and phenanthrene were above 0.1 ppb for the Amandel River, Lourens River and Fly fishing. The standard deviation of the compounds was very large and thus these values may be an overestimate of the true situation; however the concentrations found is still alarming. Wu *et al.*, in 2008 sampled river water for the analysis of PAHs, specifically naphthalene, phenanthrene, anthracene, fluoranthene and pyrene. The authors found phenanthrene concentrations of 0.38 ±0.03 ppb in unspiked river samples. These concentrations were higher than concentrations of phenanthrene found in the Lourens River, 0.154 ± 0.094 ppb.

3.3.5 Leaf sample results

The leaves data analysed by headspace gas chromatography represent estimated values and form part of vineyard leaves, berries and wine analyses (Chapter 4). The compounds that were measured ranged from 4.98 ppb to 13.5 ppm. The compounds that were present on the leaves in the highest concentrations were o-cresol (13.5 ppm), phenol (1.6 ppm), m-cresol (751.88 ppb) and p-cresol (291.7 ppb) (Table 5). The o-cresol, which was found in the highest concentrations, was only found in the Eucalyptus and apple leaf samples.

The concentrations of the compounds present on the Eucalyptus were extremely high. Due to time constraints, only one sample was analysed, and therefore there may be instrumental variance. However, considering that an internal standard was included; concentrations of o-cresol was assumed high and associated with the lipid rich leaves situated across from the stockyard. Considering that these samples were taken during the use of the creosote stockyard, the concentrations of volatile phenols found in the air was high. Plot 6 had the highest concentrations of volatile phenols present; this was the Eucalyptus leaves measured from the plantation across the stockyard. These leaves have high lipid content and may trap the compounds in the leave wax cuticle, both the lipid content and the cuticle thickness

corresponds to the season and all species have an increase from spring to summer (Emara & Shalaby, 2011). This corresponds with authors that have found lipid content of plants to be related to the accumulation of lower molecular PAHs and volatile contaminants (Simonich & Hites, 1995).

Other factors such as the age of the tree, species, leaf surface area and primary PAH content also play a role in the differences between plots as defined by Wu, *et al.* in 2008.

Table 5: Volatile phenols measured in leaves from various species surrounding the creosote stockyard.

Samples	Naph	Phenol	o-cresol	p-cresol	m-cresol	2,3 Xyl	4 EP	BTP	Ace	DBF
Weed specie	0.06834	0.245295	0	0.08574	0.989306	0	0	0.009095	0.006008	0.007063
Plums	0.001006	0.125859	0	0	0.083818	0	0.072571	0.005162	0.005999	0.007063
Plums	0.0009	0	0	0	0	0.012607	0	0.005133	0.005999	0.007063
Apple	0.008356	0.91836	0.329177	0	0	0.010432	0	0.005502	0.006	0.007063
Eucalyptus	0.00479	7.089191	67.42446	1.372808	2.686265	0.010432	0.395931	0	0.005988	0.007063
Total	*0.083392*	*8.378705*	*67.75364*	*1.458548*	*3.759388*	*0.033471*	*0.468502*	*0.024893*	*0.029993*	*0.035317*
Average	*0.016678*	*1.675741*	*13.55073*	*0.29171*	*0.751878*	*0.006694*	*0.0937*	*0.004979*	*0.005999*	*0.007063*
σ	*0.029044*	*3.046944*	*30.11667*	*0.605492*	*1.15922*	*0.006175*	*0.17185*	*0.003243*	*7.12E-06*	*1.33E-08*
Ave (ppb)	*16.67839*	*1675.741*	*13550.73*	*291.7096*	*751.8777*	*6.694284*	*93.70049*	*4.978536*	*5.998636*	*7.063326*

Wu *et al.*, 2008 furthermore investigated leaves of three tree species around a coke plant and found of naphthalene from 567± 32 to 1919 ± 263 ppt for *Cinnamomum camphora* and Pine leaves, respectively. The concentrations reported here were much higher, but used headspace gas chromatography and analysed mainly phenolic compounds.

3.4 CONCLUSION

Knowing the status of a polluted area in terms of PAH contamination is important as PAHs can contaminate soil, water, air and can be taken up by plants. These molecules are persistent and can contaminant an area for years. Furthermore particulate bound PAHs, e.g. dust bound, may contribute to health risks for human exposed and contribute to the uptake of plants. In an agricultural area the threat continues for foodstuffs products, e.g. grapes, apples, secondary products such as wines, etc. This study focused on the environmental impact of an abandoned creosote stockyard on specifically the water and soil PAHs level. The levels in water and soil found were lower than reported in previously studies and various factors are discussed for why this may be the case. However it is PAHs were still found in the soil and water and continued pollution is likely to occur via groundwater and particulate bound compounds from the industrial area. Further research must be done to conclude the environmental status of this area and bio-remediation is recommended on the contaminated soil. Continued industrial activities and vehicle emissions contribute to the presence of PAHs in the surrounding area. Inclusion of the

phenolic compounds and groundwater samples might have given a further indication of the extent of creosote pollution. Leaves had a higher accumulation of volatile organic compounds, such as naphthalene, which was reported at higher concentration than in a previous study. At the levels found in soil and water the environment is not at risk, but plants seem to accumulate these compounds, and higher concentrations may be present in the surrounding vineyards.

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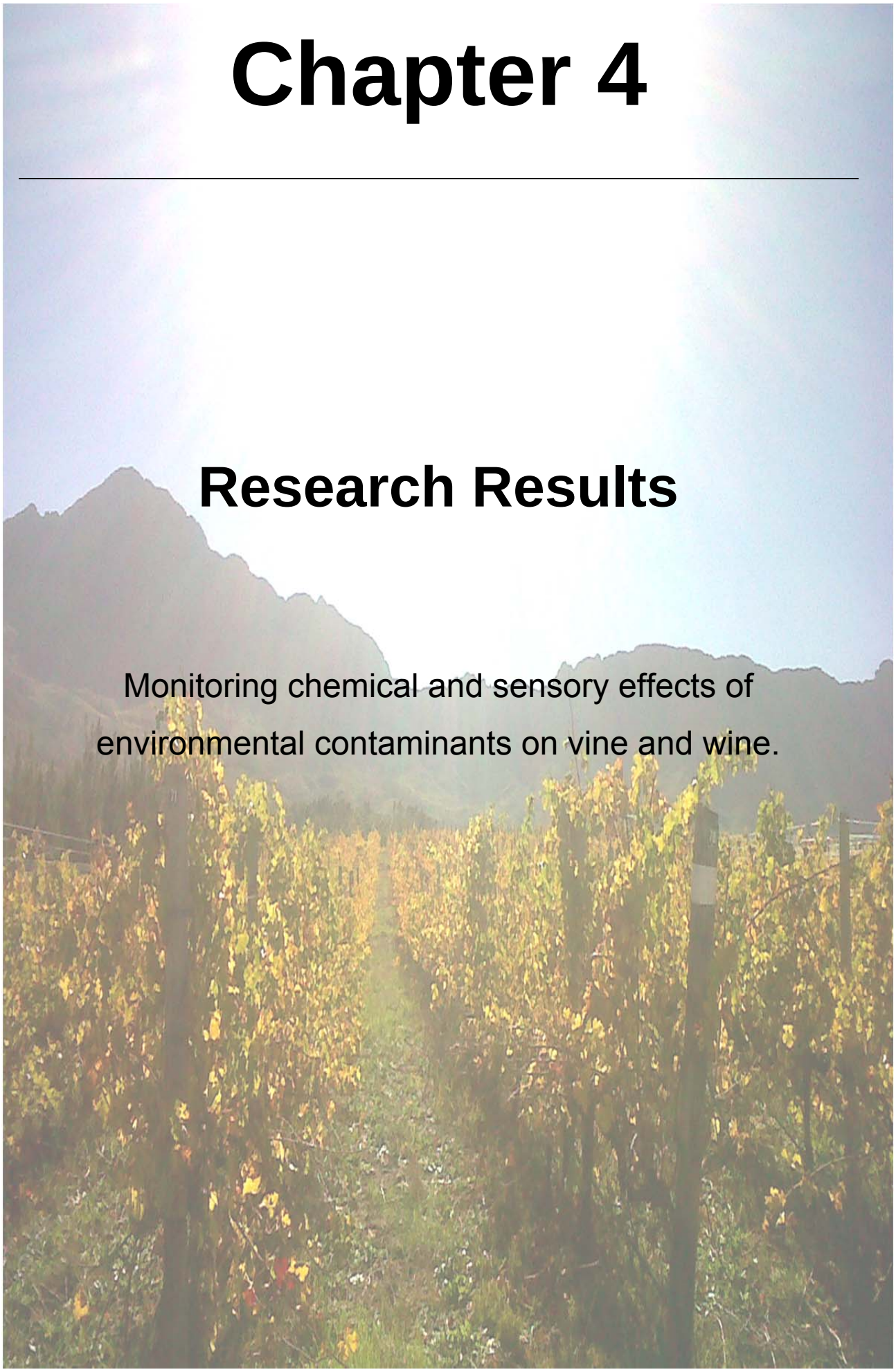
Chapter 4

Research Results

Monitoring chemical and sensory effects of environmental contaminants on vine and wine.



4.1 INTRODUCTION

Creosote is a distillation product of crude tar oil and consists of several hundred, possibly a thousand, chemicals (World Health Organization, 2004). These compounds are mainly polycyclic aromatic hydrocarbons (PAHs, 85%), N-, O-, S-containing heterocycles (5-13 %) and phenolic compounds (1-10%) (Meyer, *et al.*, 1999). The use of creosote has been severely restricted in the US, Australia, EU and Canada, due to the carcinogenic and mutagenic nature of PAHs contained in creosote treated poles (Mateus, *et al.*, 2008). The EPA has also identified 16 priority PAHs, of which benzo(a)pyrene (BaP) is used as marker molecule. The European Directive 2001/90/CE stated that benzo(a)pyrene concentrations in creosote must remain below 50 ppm by mass for treated poles (Gallego, *et al.*, 2008).

PAHs are formed during the incomplete combustion of organic material and are obtained during the distillation process of crude tar oil in the production of creosote. The creosote plant investigated in this study used high pressure tanks to impregnate the wood with creosote, after treatment the wood was stored in a stockyard, allowing the creosote to dry. During this time the treated wood emits a significant quantity of volatiles into the atmosphere, exposing the surrounding environment to these vapours. A study conducted to determine the vapours of creosote found phenol, cresols, xylenols, methyl styrene, indene, naphthalene, biphenyl, dibenzofuran, benzothiophene, quinoline, isoquinoline and fluorene to be the main components (WHO, 2004). Another study conducted on the emission from creosote-treated wood found naphthalene, toluene, m + p-xylene, ethylbenzene, o-xylene, isopropylbenzene, benzene and 2-methyl-naphthalene to be the major volatile compounds emitted (Gallego, *et al.*, 2008). The latter study found the volatile organic compounds emitted ranged from 35 $mg.m^{-3}$ to 5 $mg.m^{-3}$ from the day of treatment to 8 days after, respectively. PAHs emission ranged from 28 $\mu g.m^{-3}$ to 4 $\mu g.m^{-3}$ from the day of treatment to 8 days after, respectively.

Currently in South Africa, the use of creosote is regulated by South African National Standards (SANS) 10005 and 457. It is still applied in the built environment as well as used for trellising system construction in the agricultural sector. Poles treated with creosote, intended for the use of agricultural poles in direct contact with ground, must contain a minimum average net retention of 100 $kg.m^{-3}$ according to SANS 457-3:2000. In the Western Cape large percentage of vineyards are still trellised with creosote poles. Creosote has been reported to contain total PAH concentration of up to 85% (World Health Organization, 2004), therefore the environmental impact is often measured in PAH concentration (Agency of Toxic Substances and Disease Registry, 2003). Concerns with the use of creosote for agricultural purposes include the pollution of the air, soil and water, and,

as studies have shown, the ability of plants to accumulate PAHs (Kipopoulou, *et al.*, 1999; Camargo & Toledo, 2003; Toa, *et al.*, 2004; Wang, *et al.*, 2005; Moret, *et al.*, 2007).

Very few studies have investigated plant uptake of PAHs from creosote sources, as most studies are focussed on urban areas where plant PAH uptake is due to industrial activity and vehicle pollution. Creosote is toxic for animals and plants (Becker, *et al.*, 2001), and young vines in contact with creosote stakes showed phytotoxic reactions (Neubauer & Kasimatis, 1966). A study on soil and olives grown next to old creosote treated railway ties, showed PAH accumulation in high oil content fruit (olives), due to the lipophilic nature of PAHs (Moret, *et al.*, 2007). These authors found a total of lower molecular weight PAHs of 5-6 ppm in oils extracted from olives sampled at 1-2m distance from railway ties (source of contamination).

The uptake of PAHs in plants was found by the majority of researchers to take place in the vapour phase (retention to waxy cuticles) or by deposition of particle bound compounds (Kipopoulou, *et al.*, 1999), and the lipid content of the plant and exposed area has been shown to correlate with PAH accumulation (Wang, *et al.*, 2005; Simonich & Hites, 1995). Studies on vegetables and fruits, grown on or near sources of contamination, show the accumulation of PAHs (Camargo & Toledo, 2003) in plant material which can be a threat to human health through dietary intake. These authors found the highest mean of total PAHs in vegetables, compared with fruits; this was explained by the larger exposed area of vegetables. Furthermore grapes where found to have the highest total PAH concentration of (3.77 ppb) and individual PAHs, namely benzo(a)anthracene and benzo(k)fluoranthene.

Legislation aims to guarantee the safety of foodstuffs and although various levels have been set to create a standard in the EU, only the Czech Republic have a legal limit for PAHs in wine. The maximum limit for PAHs (the sum of benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenz(a,h)anthrachene, dibenzo(a,h)pyrene, dibenzo(a,i)pyrene, indeno(1,2,3-cd)pyrene and chrysene) is 0.5 ppb in the Czech Republic (Wenzl, *et al.*, 2006) and in general for the EU BaP has a set legal limit of 1 ppm for all dietary food (European Directive 2006/125/EC).

Studies of PAHs in wine were conducted by Chatonnet & Escobessa in 2007, who found 80-400 ppt (max. 0.4 ppb) in wines aged in new French and American barrels from different cooperages. PAHs are produced during the toasting of barrels and formation mechanisms are complex and variable. PAHs are formed from C_2 species during the pyrolysis of hydrocarbons, after formation, pyrolitic reactions, namely condensation and cyclization, may occur to form larger molecules (Chatonnet & Escobessa, 2007). The levels found in wine, due to aging in barrels, were low and not considered a health concern. PAHs found in wines and must that have been contaminated with petroleum by-products, have been found to bind with particulate matter formed during fermentation and settled in the lees. Thus PAH

pollution arising before fermentation may be partially eliminated during the settling stage of winemaking (Belin, *et al.*, 2010).

The aim of this study was to identify and monitor volatile organic compounds (VOCs) and PAHs that could potentially contaminate vines grown next to a creosote wood stockyard. Berries and leaves were sampled from the vines during the growing season, as the wax cuticle of the grapes and leaves could be a possible source of pollutant accumulation due to the lipophilic nature of the compounds. Wines (Cabernet Sauvignon and Chardonnay) were made from commercial vineyard blocks at various distances from the stockyard as well as in different styles (white, rosé and red), to determine the oenological impact on the presence of the compounds. It had been noted by winemaking staff on the estate that the vineyard blocks near the stockyard produced wines with sensorial attributes described as burnt rubber, tarry and smoky. The taints could be associated with the odours of compounds present in the volatile fraction of creosote. Thus this study attempted to identify phenols and volatile PAHs from creosote-treated wood that could contribute to the taint found in the wines. Methods were developed for the analysis of wines by headspace gas chromatography-mass spectrometry (HS GC-MS) for volatile organic compounds (VOCs), and by high performance liquid chromatography- diode array detection (HPLC-DAD) for the analysis of PAHs. Berries and leaves were analysed for VOCs by GC-MS only.

4.2 METHODS AND MATERIALS

4.2.1 Oenological treatments

Both red (Cabernet Sauvignon) and white (Chardonnay) wines were made over the 2011 and 2012 vintages. These vineyards where both adjacent to the creosote stockyard (please see layout and sampling plan in Table 1 and Appendix A), however, distances from the stockyard differed as well as the presence of spatial interferences, e.g. high trees (Blue gum forest) and rivers, in relation to the exposure to the stockyard. Harvest dates were scheduled to correlate with practice for both 2011 and 2012 vintages, and wines were made following standard experimental oenological practices.

4.2.1.1 White wine

The Chardonnay was planted in 2006, on R110 rootstock, in a 7.89 hectare (ha) block. Chardonnay were sampled and harvested for vinification from 4 sample rows (row 160, 120, 80 and 40) at different distances from the stockyard, row 160 being the closest to the stockyard and row 40 the furthest. Twenty kilograms (kg) of grapes were harvested per row. The grapes were divided into 3 to produce replicate wines per row. The wines were processed according to standard experimental winemaking conditions within the Department

of Viticulture and Oenology. The must was fermented until dry (<5 g/l fructose + glucose) at 15˚C and had skin contact of less than 12 hours at 4˚C before inoculation. Bentonite was added after fermentation, instead of before, to decrease the loss of wine and aroma. Bentonite was not believed to have an effect on PAHs concentration due to a study done on fining agents and their effect on the taint associated with the compounds of interest. After alcoholic fermentation, 35 ppm free SO_2 was added and cold stabilization took place before bottling.

4.2.1.2 Red wine

Four blocks of Cabernet Sauvignon were planted in 2003 on rootstock R110, except for the third block, planted on 101-14 Mgt. The eastern side of block 2 and 3 are affected by leaf-roll virus, possibly due to the exposed roads that contribute to air-borne dust particles, where the western side of the blocks are protected from the road by trees. Dust particles are known to participate as a vector for viruses (Walton, 2007) and have a direct influence on the quality and crop load of the fruit obtained from these affected areas (Goussard, *et al.*, 2004). PAHs may bind to particulate matter in the air, e.g. dust, and thus these blocks are also exposed to particulate PAH contamination (Dahlgren, *et al.*, 2003). The sizes of blocks 1 to 4, were 3 ha, 2.82 ha, 2.75 ha and 2.88 ha, respectively.

Four 20kg batches of grapes were harvested from blocks 1 to 4. From each block, two rows were allocated by the farm for sampling. In 2011 the grapes, four ±20kg batches from each block, were homogenised before redividing it into 4 equal batches for treatments A to D (Table 1). In 2012 however, the grapes were not homogenized but the two sample rows were divided in half, to form 4 plots per block. This was done to include more of the variation within the block, mainly due to the leaf-roll virus, and to identify areas of contamination. These four plots were used for the collection of the 20kg grapes for treatments A-D for each block. The plots were automatically randomized (MS Excel) for each block and thus the treatments were applied to a different plot for every block. The differences in harvesting strategies did not have an effect on the statically analysis, as both vintages were processed simultaneously within a single data set.

The treatments (A and C) applied with the aim of finding a practical approach for the winery to lower the taint, until environmental remediation takes place. Due to the lipophilic and particulate nature of PAHs accumulation in plants (Moret, *et al.*, 2007), skin contact duration (rosé vs. red) and washing of grapes with water for removal of particle bound PAHs, were the treatments aimed specifically for practical application by the winery. Treatment B is the control treatment and is considered standard red wine making practice, whilst treatment D aims to remove the waxy layer on the berries, and the PAHs bound to the skins. Treatment D is considered an extreme scientific treatment and was only chemically analysed

due to the toxic acetonitrile (ACN) residue in the wines. The treatments were each made from a batch of ± 20 kg grapes which was further divided into triplicates. The experimental wines made could not have been contaminated with *Brettanomyces sp.* because all wines where made under sterile conditions in a controlled environment. Therefore all phenols measured were assumed from the creosote source.

Table 1: Red wine treatment layout

Wine Style	Block	Treatment	Rep	Standard procedure
CS Rosé	1	A	1,2,3	4-6 hours skin contact, inoculated with Anchor NT16
CS Rosé	2	A	1,2,3	Alcohol fermentation until dry at 15˚C
CS Rosé	3	A	1,2,3	Addition of SO2 and bentonite AF
CS Rosé	4	A	1,2,3	Cold stabilization at -4˚C before bottling
Cabernet Sauvignon	1	B	1,2,3	Must inoculated with Anchor NT16, AF on skins for 8 days
Cabernet Sauvignon	2	B	1,2,3	Alcoholic fermentation until dry at 25˚
Cabernet Sauvignon	3	B	1,2,3	Lysozyme and SO2 additions after AF to prevent MLF
Cabernet Sauvignon	4	B	1,2,3	Cold stabilization before bottling
CS, treated grapes	1	C	1,2,3	Procedure same as treatment B but with water washed grapes
CS, treated grapes	2	C	1,2,3	
CS, treated grapes	3	C	1,2,3	
CS, treated grapes	4	C	1,2,3	
CS, treated grapes	1	D	1,2,3	Procedure same as treatment B but with ACN washed grapes
CS, treated grapes	2	D	1,2,3	
CS, treated grapes	3	D	1,2,3	
CS, treated grapes	4	D	1,2,3	

Treatment: A) Rosé wine, with less than 4 to 6 hours skin contact, fermented at 15˚C until dry (<5g/l fructose + glucose) B) control dry red wine, fermented on the skins for 8 days C) dry red wine, berries washed with 1 litre of water before crushing (to remove dust particles (particulate bound pollutants) D) dry red wine, made from berries washed with 1 litre of acetonitrile to remove wax cuticle before crushing.

The same yeast strain, Anchor NT16, was used for all Cabernet fermentations, which was both suited for cool rosé fermentations, as well as red wine fermentation. Treatment A was fined with bentonite for protein stability and sulphured to 35 ppm free SO_2 before cold stabilization and bottling. Treatment B, C and D were pressed after the 8th day of fermentation and allowed to ferment until dry (<5g/l glucose + fructose). No malolactic fermentations were done on these treatments to avoid any phenol production. Therefore they were sulphured to free 50 ppm SO_2 after alcoholic fermentation and lysozyme was added to prevent spontaneous malolactic fermentation. The red wines were cold stabilised before bottling.

4.2.2 Sampling of grapes and leaves

4.2.2.1 Berries

Berries were sampled from four blocks of *Vitis vinifera* cv. Cabernet Sauvignon. The berries change in morphology over the growing season, January to April in the Southern Hemisphere. Therefore the grapes berries were sampled twice during the ripening stage from two sample rows per block, representing duplicates per block. The motivation for the sampling being that the wax layer decreases in thickness post-véraison (Grncarevic & Radler, 1971) and could potentially accumulate organic pollutants which are highly lipophilic. The berries were sampled at two different sugar levels at véraison (14˚B) and post véraison (18˚B) into glass containers and kept frozen, -4˚C, and protected from light until analysis.

4.2.2.2 Leaves

Vineyard leaves from the four Cabernet Sauvignon blocks facing the creosote stockyard were sampled post-harvest over the two growing seasons, 2011 and 2012. Leaves were sampled to determine if the volatile compounds accumulated on the wax layers. From literature it is known that the leaf wax layer is a tenth of the thickness of the berry wax layer (Rosenquist & Morrison, 1989). Two sample rows per block were sampled as duplicates per block. Leaves were sampled into glass containers and kept frozen at -4˚C, and protected from light, as to protect the volatile organic compounds from volatilization and photo-degradation.

4.2.3 Sensory Analysis

All sensory analysis was done in isolated booths in a well ventilated sensory lab at a temperature of 20˚C±2˚C. The 25 ml wine samples were presented at random in covered ISO standard glasses. The samples were each marked with a random 3 digit code. Yogurt, water and crackers were given with each sample set, to aid in palette cleansing, as astringency was analysed, along with “burnt rubber” or “tar”, which have a carry over effect. In 2011 the wines were analysed by a trained panel consisting of 11 individuals (9 female panellists and 2 male panellists) all whom had previous experience in sensory descriptive wine analysis and were either staff, or postgraduate students of the DVO or IWBT. In 2012, the 11 panellists, again from the working and postgraduate environment of the DVO, were 4 males and 7 female panellists.

4.2.3.1 Panel training

The training was done in 1 hour sessions, 2 to 3 times a week. This was arranged according to the availability of the panel. The samples were each tasted in triplicate within one session; this was achieved by randomizing the samples according to the Latin-square. Two-way

ANOVA analysis was done, because of the layout of the tastings. The ANOVA was carried out using Microsoft Excel© 2007, PanelCheck® and STATISTICA. The panel firstly underwent general training, which consisted of language/descriptive development. During these 5 general training sessions, panellists were trained on the recognition of taints at different levels. For the aims of this specific study, further training was done before final analysis to determine panel consensus on the attributes used. The sensory assessments were carried out in 4 sessions, the first was a trail test, and the sessions after that was for white, rosé and red wine analysis respectively (J.Brand, personal communication, 2012).

Compounds that were used in the training sessions were 4 ethylphenol, 3,4-xylenol, o-cresol, trichloro-anisole and dimethyl sulphide (Table 2). Concentrations were chosen above detection threshold values for odours to aid as a screening for the sensitivity of the panellists. The dimethyl sulphide was much lower as was reported by other authors, e.g. 10-160 ppb (Escudero, *et al.*, 2007) however wines were easily identified according to "cabbage" and "onion/garlic" attributes at 4 ppb. The 4 ethylphenol concentration was much higher as the reported concentration, but was adapted to be easily identifiable by the panel.

Table 2: Levels of compounds spiked during training.

Compound	Attribute	Spiking level	Odour threshold	Reference
Dimethyl sulphide	Sulphur/vegetative	4 ppb	10 - 160 ppb	Escudero, *et al.*, 2007
o-cresol	Phenolic	50 ppb	62 ppb	Parker, *et al.*, 2012
3,4 Xylenol	Phenolic	740 ppb	-	Brand, p.c., 2012
4 Ethylphenol	Phenolic/Brett	750 ppb	440 ppb	Escudero, *et al.*, 2007
Trichloro-anisole	TCA/Cork	2 ppt	1-5 ppt	Mazzoleni & Maggi, 2006
Aluminium Sulphate	Astringency	0.3 g/l	-	Brand, p.c., 2012
Creosote block	Tarry	n/a	-	
Fresh berries	Fruitiness	n/a	-	

The samples were prepared by adding the concentration to commercial neutral wine. The stock solutions used to spike the wine was made up from the pure compound in ethanol. A second training session, with samples to be analysed and reference standards, was done along with a trail test to analyse panel consensus. In a third session feedback was given to the panel on the trial test so that panellists could calibrate themselves before the analysis.
The white wines were tasted in triplicate in one session, with breaks after each sample set. Thereafter training commenced on the Rosé wines, because the taint is similar to the taint found in white wine, only one training session was needed to achieve panel consensus. During this training a trail test and discussion took place. The Rosé wines were tasted in black glasses in 2012, due to the colour differences between the treatments. In 2011 this

was not needed because the differences in colour were not significant. For the red wine training, samples as well as standards were given for the predominant attributes, as more of these were found in red wine (Table 2). Lastly the wines were analysed in triplicate in one session. Breaks were taken in between sample sets to prevent carry over of taints.

4.2.4 Headspace gas chromatography-mass spectrometry (HS GC-MS)

Headspace GC-MS was used for the analysis of wines, berries and leaves for the detection of 12 volatile phenolic compounds found in the volatile fraction of creosote emissions. This method was developed, as no method was found in literature for the detection of these compounds in wines. The internal standard was selected as 3-octanol, it has a similar structure as the phenols, but neither elute with any of the compounds nor is commonly found in wine samples. Sample preparation commenced as close to analysis as was possible, due to the volatile nature of the compounds. The compounds analysed include naphthalene, o-cresol, m-cresol, p-cresol, 2,6-xylenol, 3,4-xylenol, 2,3-xylenol, acenaphthene, dibenzofuran, phenol, 4-ethylphenol, benzothiophene (Table 3). The compounds were selected for analysis on the basis of the odorous nature and their possibility to contribute to the taint in wines.

4.2.4.1 Reagents

The pure compounds of 3-octanol, naphthalene, m-cresol, o-cresol, 2,3-xylenol, 4 ethylphenol, acenaphthene were purchased from Sigma- Aldrich (Steinheim, Germany), 4 ethylguaiacol from SAFC (St Luis, MO), p-cresol and dibenzofuran from Fluka Analytical (Steinheim, Germany), 2,6-xylenol and 3,4-xylenol from Riedel-de Haën (Hanover, Germany) and lastly m-cresol and benzothiophene from Merck (Hohenbrunn, Germany). Pure compounds were weighted into one 50 ml volumetric flask, and dissolved in synthetic wine. This standard mixture was used for making calibration standards.

The synthetic wine was made as follows: 240 ml absolute ethanol in 1.6 litre deionised water (12% v/v), ±5 g tartaric acid dissolved in ethanol water (pH of 2.5), the pH is then adapted to 3.5 with NaOH pellets, after all compounds are dissolved, with the use of a magnetic stirrer, the level was filled to 2 litre mark with deionised water.

4.2.4.2 Calibration

The standard mixture used for spiking the 8 point calibration consisted out of 13 compounds weighted from the pure compound and dissolved in synthetic wine. The calibration curve ranged from 0.001 ppm to 1 ppm. The internal standard, 3-octanol, was added at 0.1 ppm to 5 ml of each calibration concentration in synthetic wine. The internal standard was used for calibration and quantification and represented a mid-range concentration.

Table 3: GC-MS compounds chemical and analytical properties

Compounds	Monitoring Ions	± RT(min)	Odour	Odour threshold(OT)	OT in wine	Ref[a]
Naphthalene	128[b]	27.78	Mothballs	84ppb		2
Phenol	66,94[b]	30.416	sickeningly sweet, irritating	5 500 ppb	2-4 ppm	1
Acenaphthene	154[b]	33.014	Tarry	80 ppb	3-17ppt	3,6
4 Ethylguaiacol	137,152[b]	30.85	Smoky, clove-like	25 ppb	33 ppb	1,5
2,6 Xylenol	107[b]	29.3	medicinal Phenolic	400 ppb	570 ppb	1,5
Dibenzofuran	139,168[b]	34.8		150 ppb		1
o-Cresol	107,108[b],122	30.323	musty, Phenolic aftertaste		30 ppb	4
p-Cresol	107,108[b],122	31.32	Phenol-like	55-100 ppb	64 ppb	4
m-Cresol	107,108[b],122	31.42	dry, tarry, medicinal-leathery	0.3 ppb	20 ppb	4
2,3 Xylenol	107,122[b]	32.13		0.7ppb		1
4 Ethylphenol	107,122[b]	32.469	woody-Phenolic, medicinal and sweet	42-130 ppb	440 ppb	1,5
Benzothiophene	134[b]	28.611				1
3,4 Xylenol	107,122[b]	33.281	flat dry	1200 ppb		1

(a): References: (1) Fenaroli's handbook of flavor ingredients, 6th edition. George A. Burdock; (2) http://www.atsdr.cdc.gov/toxprofiles/tp67-c1.pdf; (3) http://www.toronto.ca/health/pdf/cr_appendix_b_pah.pdf; (4) Parker, *et al.*, 2012 (5) Escudero, *et al.*, 2007(6) Chatonnet & Escobessa, 2007

(b) Quantifier ion

4.2.4.3 Sample preparation

Wines

The wines (5 ml) were placed in a 20 ml vial containing NaCl (1 g), to facilitate the volatilisation of the compounds into the headspace. The internal standard (3-octanol) was added at 0.1 ppm before it was sealed with a septum-type cap. The samples were agitated by Vortex before analysis. Head Space-Solid Phase Microextraction (HS-SPME) was used for online extraction of the compounds.

Berries

The wine sample preparation method was adapted for berries. Skins and pulp were separated to determine the concentrations present in both. Ten to twelve berries, depending on size, were peeled, separating the skins for the pulp and pips. The skins were then homogenised with 5 ml methanol/water (30/100) solution. The skin-methanol solution was transferred into a glass vial containing 1 g of NaCl salt and a magnetic stirrer bar before addition of 3-octanol at 0.1 ppm. The pulp and pips were homogenised separately and weighed to 5 g in a glass vial with 1 g of NaCl and magnetic stirrer bar and addition of 3-octanol at 0.1 ppm. The samples were stirred on a magnetic plate before analysis.

This method was developed using berries exposed to creosote blocks, for several time periods, in glass jars, before analysis. The berries prepared as above discussed, all showed to contain the volatile phenols. The same SPME method was used for berries, wines and leaves.

Leaves

The leaf preparation method was adapted from the berry procedure. Leaves were homogenised with 10 ml of methanol/water (30/100) and 5 g was weighed in a glass vial containing 1 g of NaCl salt. The method was tested by exposing 5 g leaves to creosote blocks for 6 hours, thereafter sample preparation commenced, according to the above procedure, before analysis. The leaves showed the presence of the compounds in high concentrations versus control leaves exposed to no creosote emissions.

Head Space-Solid Phase Microextraction (HS-SPME) was used for online extraction of the compounds using a 65 µm polydimethylsilxane/divinylbenzene (PDMS/DVB) coated fibre (pink). The samples in the vials were allowed to equilibrate for 1 min at a temperature of 50ºC in the CTC auto sampler incubator and after this equilibration time, the fibre was exposed to the headspace for 20 min at 50ºC. After sampling, desorption of the volatile compounds from the fibre coating was carried out in the injection port of the gas chromatography-mass spectrometry (GC-MS) for 10 min in splitless mode. The temperature of the injection was maintained at 250ºC. After desorption, the fibre was heated for 10 minutes in the heating chamber maintained at a temperature of 250ºC before the next run to avoid cross contamination from the previous run.

4.2.4.4 Instrumentation

The separation of the volatile phenols was performed on a gas chromatograph, Agilent technologies network GC system, model 6890N, coupled with an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD), model 5975B, (Agilent Technologies Inc., Palo Alto, CA). The GC-MS system was coupled with a CTC Analytics PAL auto sampler. Separation of the volatile phenols of interest was performed on a FFAP column (60m × 250 µm × 0.50 µm), model J&W 122-3263, from Agilent Technologies. Helium was used as a carrier gas maintained at a flow rate of 1 $ml.min^{-1}$ throughout the analysis. The injector was operated in splitless mode and maintained at 240˚C. The oven programme was operated at an initial temperature of 40˚C held for 3min, thereafter ramped to 100˚C at 5 ˚C/min held for 2min and again ramped to 180˚C at 20 ˚C/min held for 5min and then finally ramped to 240˚C at 20 ˚C/min and held for 15min. The total run time of the method was 44 minutes. The MSD was operated in Scan and single ion monitoring (SIM) mode to obtain the data. The SIM data was used for quantification purposes. The scan parameters were from 25 for

low mass and 650 for high mass. The MS source and the quad were maintained at 240˚C and 150˚C respectively and the transfer line temperature was maintained at 280˚C. Compounds were identified using GC-MS retention times and identification of the mass spectra with NIST05 spectral library collection (L. Mokwena, personal communication, 2012)

4.2.5 High performance liquid chromatography-diode array detection (HPLC-DAD)

The analytical instrumental method was developed for the quantification of the 16 priority EPA PAHs in wine, soil and water. Not all of the compounds were detectable at the lower calibration levels, therefore, for the purpose of this study, the PAH analysis was limited to 12 compounds, as listed in Table 4.

4.2.5.1 Reagents

The solvents used for sample preparation and instrumentation were HPLC grade acetonitrile from Merck (Germany), cyclo-hexane (Merck), and Na_2SO_4 anhydrous purchased from Sigma-Aldrich (Steinheim, Germany). The pure compound PAHs (Table 4) and internal standard, 2-ethyl anthracene, were purchased form Sigma-Aldrich (Steinheim, Germany), except for benzo(a)anthracene, anthracene and chrysene were from Fluka Analytical and benzo(g,h,i)perylene from Supelco (Bellafonte, PA, USA).

4.2.5.2 Calibration, method validation and recovery studies

The 6 point calibration was done in synthetic wines with the concentration range of 5, 10, 25, 50, 100 and 250 parts per billion (ppb). The calibration samples were prepared by directly spiking model wine, with internal standard (2 ethyl acenaphthene at 40 ppb), before sample preparation with the developed LLE method. Equations were obtained by plotting the ratio (peak area PAH/peak area internal standard), against the concentrations (x-axis) and the coefficient of determination (R^2) was calculated (Table 4). The recovery study was done in triplicate for synthetic, white and red wine at both 25 ppb and 100 ppb. Recoveries obtained for each compound are summarized in Table 4. The limit of quantitation (LOQ) and limit of detection (LOD) were also calculated using the instrumental signal to noise ratio of the compounds at of 10 for LOQ and 3 for LOD. The concentrations are given in Table 4 in ppb. Acenaphthylene, acenaphthene, naphthalene and fluorene could not be detected at all the concentrations. Due to the volatility of some of these compounds, it was assumed that the compounds were either lost by volatilization or during another sample preparation step. Acenaphthene and naphthalene were however included in the GC-MS analysis. The recoveries obtained for the compounds were very good considering the low concentration range.

Table 4: HPLC-DAD method development

					LOD	LOQ	Recovery white wine		Recovery red wine	
Compounds	RT	λ	Calibration	R^2	ppb	ppb	25ppb	100ppb	25ppb	100ppb
Benzo(a)Anthracene	16.768	270	y=0.006x + 0.001	1	0.5226	1.7422	97%	99%	110%	112%
Benzo(a)Pyrene	19.41	254	y=0.008x - 0.011	0.999	0.3916	1.3055	102%	108%	118%	126%
Benzo(b)Fluorene	18.793	254	y=0.004x + 0.006	0.999	0.6237	2.0790	96%	104%	111%	121%
Chrysene	16.502	270	y=0.010x + 0.018	1	0.2650	0.0883	97%	101%	113%	118%
Acenaphthene	8.88	230								
Acenaphthylene	10.874	230								
Anthracene	12.491	254	y=0.018x + 0.076	0.998	0.1307	0.4355	87%	100%	82%	86%
Fluoranthene	13.93	270	y=0.002x + 0.002	0.999	1.6043	5.3476	88%	91%	99%	106%
Napthalene	7.48	230								
Phenanthrene	11.873	254	y=0.009x + 0.02	0.998	0.4016	1.3387	115%	115%	108%	87%
Pyrene	14.376	230	y=0.006x + 0.001	0.999	0.6303	2.1008	97%	88%	110%	98%
Fluorene	11.068	254	y= 0.002x + 0.001	0.987			116%	187%	940%	54%

4.2.5.3 Sample preparation

HPLC DAD was used for the determination of PAHs in wines made from various plots and treatments. A liquid-liquid extraction (LLE) sample preparation procedure was developed for the isolation of these compounds from wines. Twenty-five ml of wine, with the added internal standard at 40 ppb (50 µl of 20 ppm stock of 2 ethyl anthracene) was extracted using 3 ml cyclo-hexane as the solvent. The samples where placed in an ultrasonic bath for 20 minutes, and shaken every 5 minutes. After extraction the samples were allowed to reduce in vapour pressure by cooling at -20˚C for 15 min. The surface organic layer was then transferred into smaller tubes with Pasteur pipettes. A second extraction on the sample was done using another 3 ml cyclo-hexane and following the steps as before. The second organic layer was added to the first and the emulsion was then centrifuged twice for 5 minutes, until the organic layer separated completely. The organic layer was then transferred to clean glass tubes containing anhydrous sodium sulphate, enough to cover the bottom of the tube. The contact area with the sodium sulphate was increased by vortex, enabling the removal of any possible wine contamination. The samples were transferred from the salt into a final glass tube and then evaporated until dryness at 40˚C under a gentle stream of nitrogen in a sample concentrator (Techne DB-3 series Driblock (UK)). Acetonitrile (1 ml) was added and the PAHs were dissolved, facilitated by vortex agitation. Finally the samples were sterile filtrated trough 0.45 µm nylon filter (Agela Technologies) and 1 ml syringe (Surgi-plus) into amber HPLC grade vials, and stored at -20˚C until analysis (A. Buica, personal communication, 2011).

4.2.5.4 Instrumentation

Analysis for PAHs in environmental samples, soil and water, and wine were done using an Agilent 1260 Infinity HPLC-DAD instrument from Agilent Technologies Inc. (Palo Alto, CA). The system was equipped with a C18 reverse-phase column (Poroshell® 120), with diameters 4.6 × 50 mm and i.d. 2.7 micron particle size (Agilent). The column was maintained at 25˚C during analysis. The mobile phases were ultrapure water (A) and acetonitrile (B), and a flow rate of 1 ml/minute was maintained. The chromatographic conditions were programmed as follows: 0-2 minute of 40% B, 2-22 minutes of 40-80% B, 22-26 minutes of 80-100% B and 26-29 minutes of 100% B. The total run time, including reconditioning, was 34.5 minutes. The Diode Array Detector (DAD) was programmed to monitor readings form 190-400 nm for the identification of PAHs. Quantification was done at 230, 254 and 270 nm. Chemstation® software from Agilent (Palo Alto, CA) was used for identification and data analysis.

4.2.6 Statistical analysis

Sensory and chemical data was analysed statistically to determine wine treatment effects. PanelCheck® was used to determine the panel performance and product effect, tucker plot and bi-plot analysis was done. Tucker-plots were used to determine the panel agreement for each attribute and product effect histograms were constructed to determine the significance of the attributes. Furthermore bi-plots, principle component analysis (PCA), were used to determine the correlation between wines and attributes. All PCA plots were done with LatentiX® software. The data was also treated with one-way analysis of variance (ANOVA) at a 95% confidence interval. For the sensory data, block effects and treatments were looked at for the 3 styles of wine across two vintages, whilst for chemical data treatment, block, vintage and interaction effects were analysed per compound as determined by HS SPME GC-MS and HPLC-DAD analysis. Least significant difference (LSD) was performed to identify significant difference for factors. The ANOVA analysis was done using Statistica software (Statistica 10.0, Statsoft, USA).

4.3 RESULTS AND DISCUSSION

The results obtained were from wine, berry and leaf samples of *Vitis vinifera* cv. Cabernet Sauvignon and Chardonnay made and sampled over the 2011 and 2012 vintages. Inevitable variance persists between vintages in terms of climatic conditions and, in the case of this study, the activity of the stockyard. The creosoting plant closed at the end of 2010 and the stockyard remained in use until the end of 2011. Thereafter only other industrial activities, e.g. vehicle and factory emissions could contribute to VOC and PAH

contamination in the vineyard. Furthermore, 2011 was a much warmer and earlier season for Cabernet Sauvignon than 2012, which was a temperate and longer season.

4.3.1 Sensory results

4.3.1.1 Panel performance 2011

The panel performance was tested with PanelCheck® using guidelines as recommended by Tomic, *et al.*, in 2010. All the sensory data for white, rosé and red wines were combined and used to construct the Tucker plot, product effect and bi-plots.

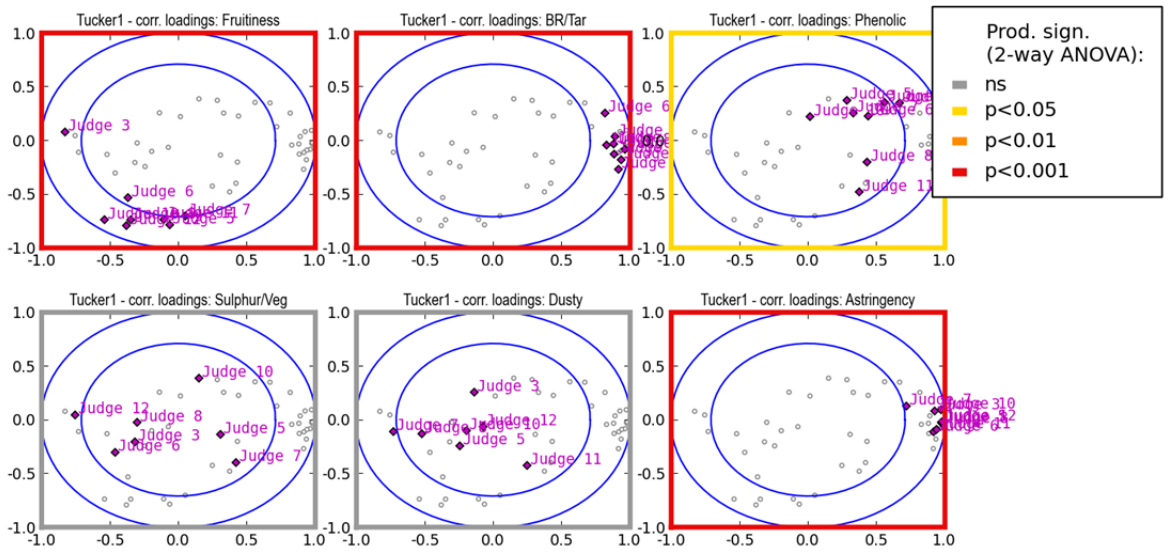


Figure 1: Tucker plot: Panel agreement on attribute description 2011 for all wines (PanelCheck)

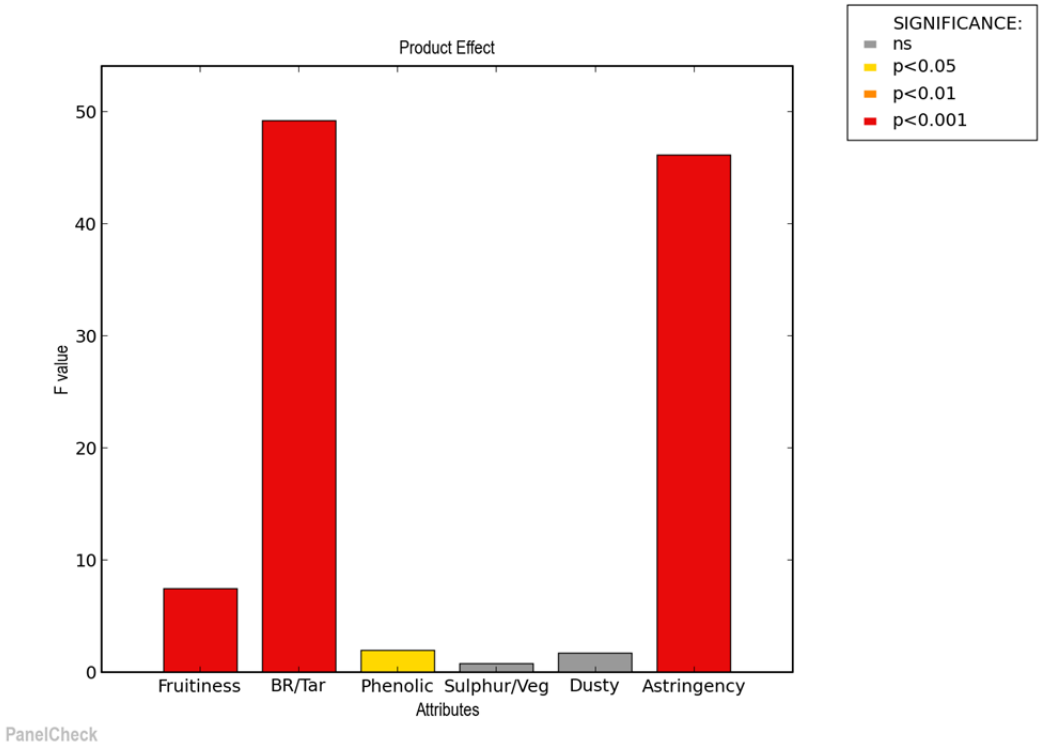


Figure 2: ANOVA product effect showing BR/Tar attribute at a significance p value of 0.001

The above data shows that the panel had satisfactory agreement at a significance level of 0.1% for the burnt rubber/tar, fruitiness and astringency attribute. The panel obtained a significance level of 5% for the phenolic attribute. Sulphur/vegetative and dusty attributes were not significant, thus ANOVA analysis is not included for these attributes. These plots were analysed to determine the panel performance with all the data obtained from the white, rosé and red wines tastings.

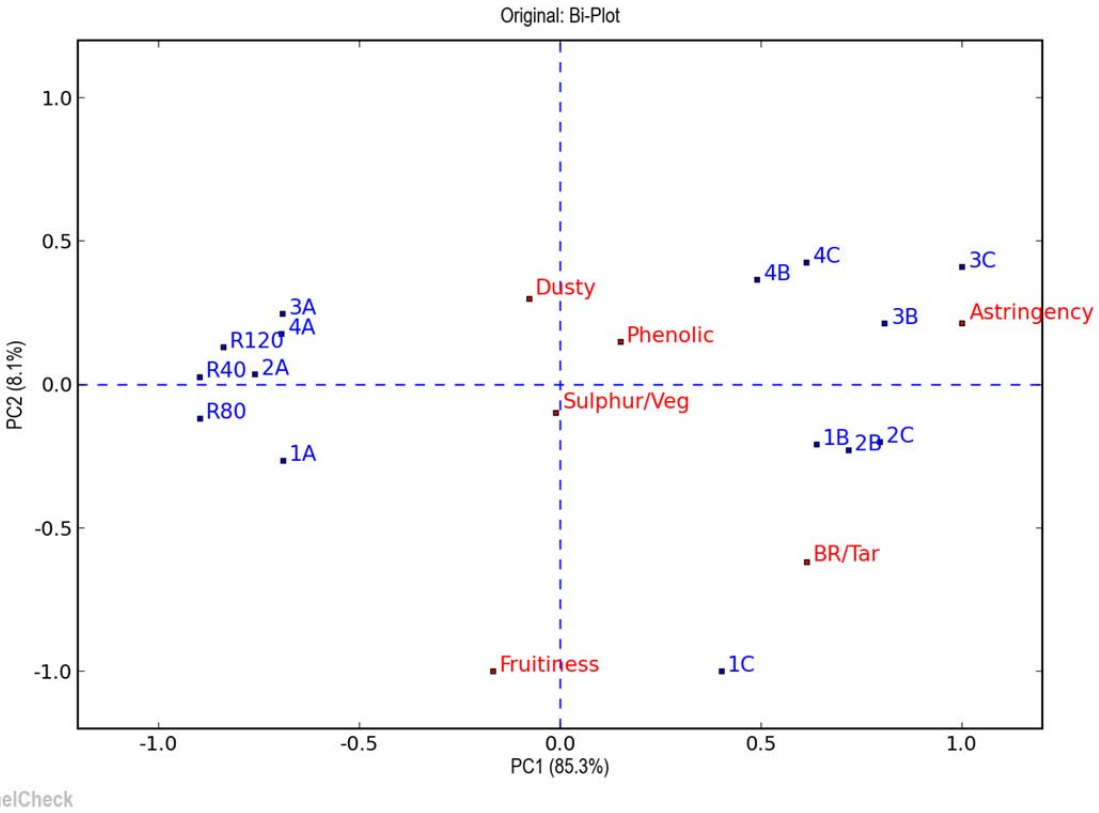


Figure 3: Bi-plot: correlates treatments (including blocks and oenological treatments) with attributes found by the panel. R40, R80, R120 and R160 is the Chardonnay wines made from 4 rows, 1 to 4 refers to the Cabernet Sauvignon blocks and A-D refer to the wine-making treatments applied to the Cabernet Sauvignon.

This bi-plot illustrates the association of all wines made from the affected blocks. The explained variance for the first two principle components (PC) is 93.4%. Each sample given is the average of the 3 replicates analysed. The white and rosé wines are split from the red wine treatments by PC1. Even though rosé wines, treatment A, were made from the same blocks as the red wine treatments (B and C). Burnt rubber/tar, astringency and to a lesser extent the phenolic descriptors were mostly associated with the red wines. This suggests that skin contact practices were correlated with sensorially significant taint attributes. Block 1 and 2 were mostly associated with the burnt rubber/tar attribute, whilst block 3 was associated with astringency and block 4 with phenolic attributes. The white and rosé wines

were not associated with the attributes contributing to the taint in effected wines, and therefore the subtle chemical differences were investigated further with GC-MS analysis.

4.3.1.2 Analysis of Variance (ANOVA) 2011

The ANOVA least square plots (Figure 4 to 7) show the block effect in further detail for the significant attributes. The 'fruitiness' attribute did not show an increase in perceived fruit character of the wines with distance increases from the source of pollution, as expected. Block 1 was the highest in fruitiness, with block 3 significantly (5%level) lower, block 2 and 4 were not significantly different from either block 1 or 3 (Figure 4). The ANOVA analysis done on the 2011 sensory data shows a decrease in the burnt rubber/tar attribute from block 1 to 4, block 4 being significantly lower than blocks 1, 2 and 3 (Figure 5). The astringency effect does not correlate with the other attributes- for block 2 and 3 is perceived as the highest, whilst block 1 and 4 is significantly lower than the other blocks (Figure 6). Both the trends shown by the fruitiness and astringency attributes could possibly be due to the leaf-roll virus that is present in block 2 and 3, as this virus is responsible for acid/sugar imbalances and influences the development of phenolic ripeness (Goussard & Underhay, 2004), thus having an effect on fermentation and having more astringent wines as result. During further studies compounds, e.g. phenol, contributed to the sweet and fruitiness of wines and may contribute to the blocks associate with fruitiness (Panzeri, *et al.*, 2012). Astringency could also be influenced by wine making practices and unripe harvesting, but since all wines were treated the same this was not regarded as a factor. Therefore it is concluded that complex factors influence the fruitiness of the wine. The perceived clean samples, however does increase with the distance from the creosote stockyard, with the furthest block 4 significantly different from blocks 1, 2 and 3 (Figure 7).

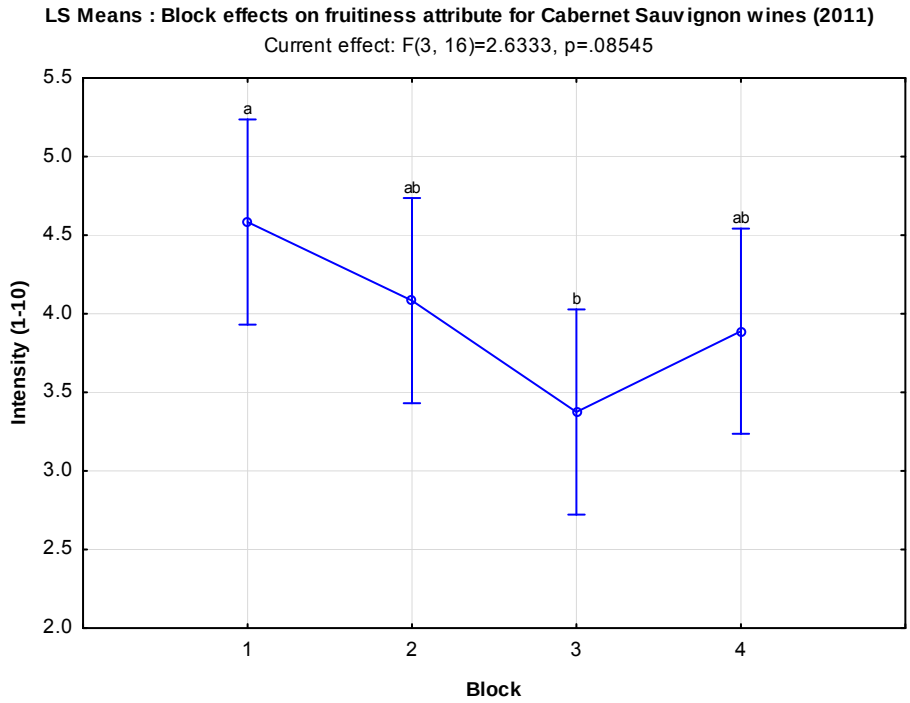


Figure 4: ANOVA Least square means graph of fruitiness attribute trend in Cabernet Sauvignon blocks.

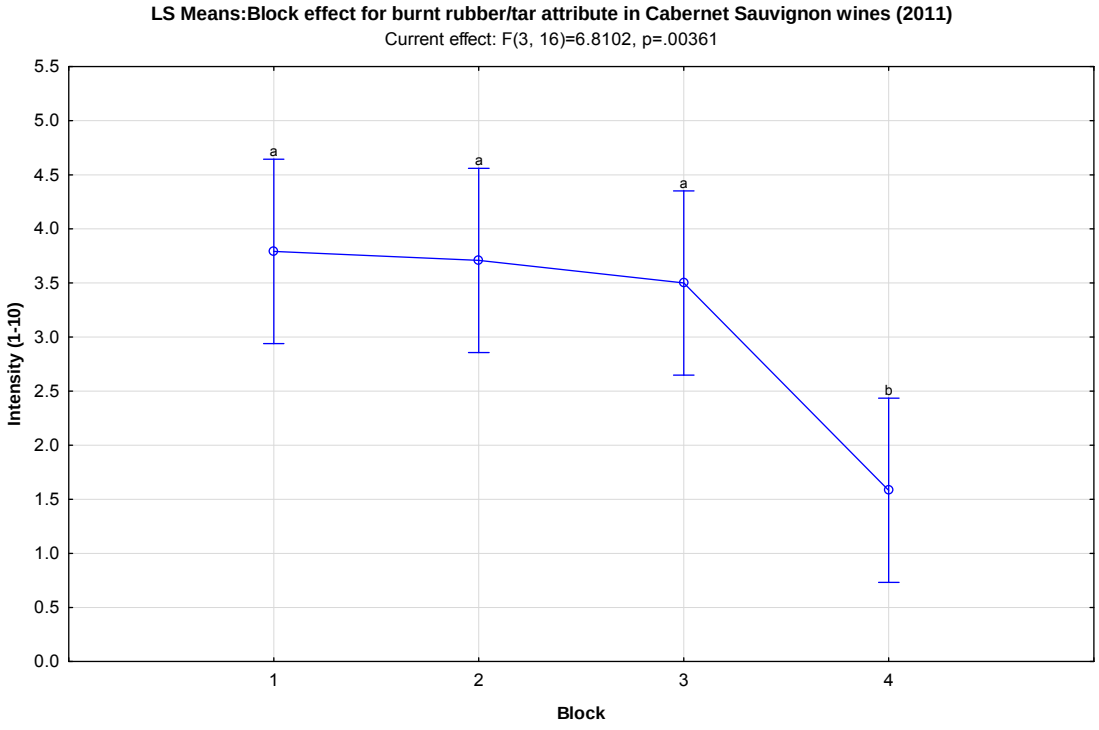


Figure 5: ANOVA Least square means graph showing burnt rubber/tar attribute across the Cabernet Sauvignon blocks in 2011.

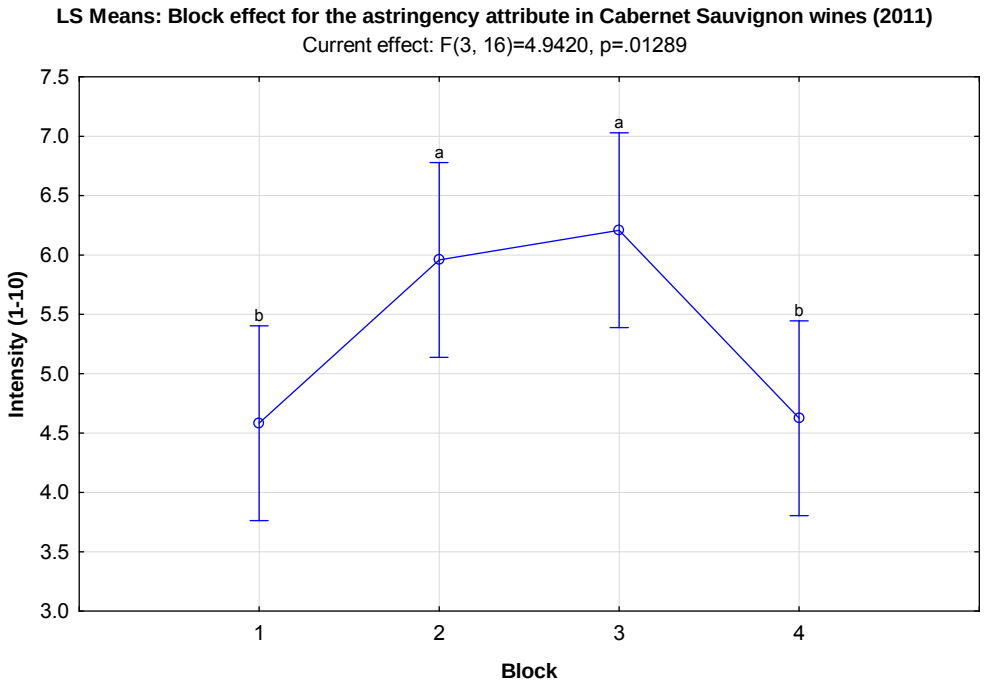


Figure 6: ANOVA least square means shows astringency trend in Cabernet Sauvignon blocks adjacent to stockyard.

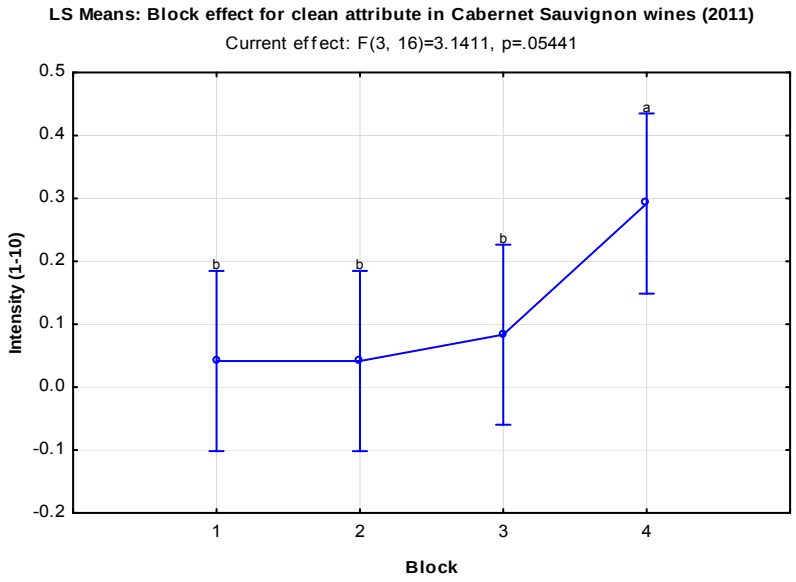


Figure 7: ANOVA least square means graph shows increase in perceived clean samples as distance from stockyard increase.

4.3.1.3 Panel performance 2012

The panel performance was analysed with PanelCheck® and again all data was combined for the construction of Tucker plot, Bi-Plot and Product effect. A Bi-plot of only the red wines was also constructed in 2012.

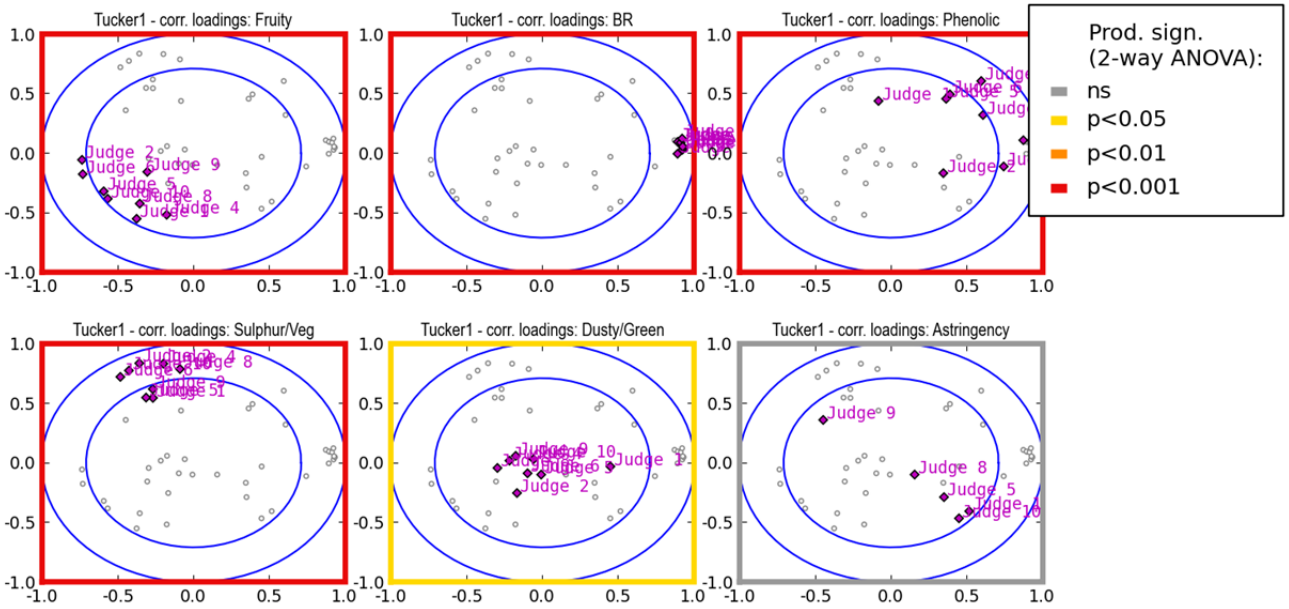


Figure 8: Tucker plots: Panel agreement on attribute descriptors 2012 (Panelcheck)

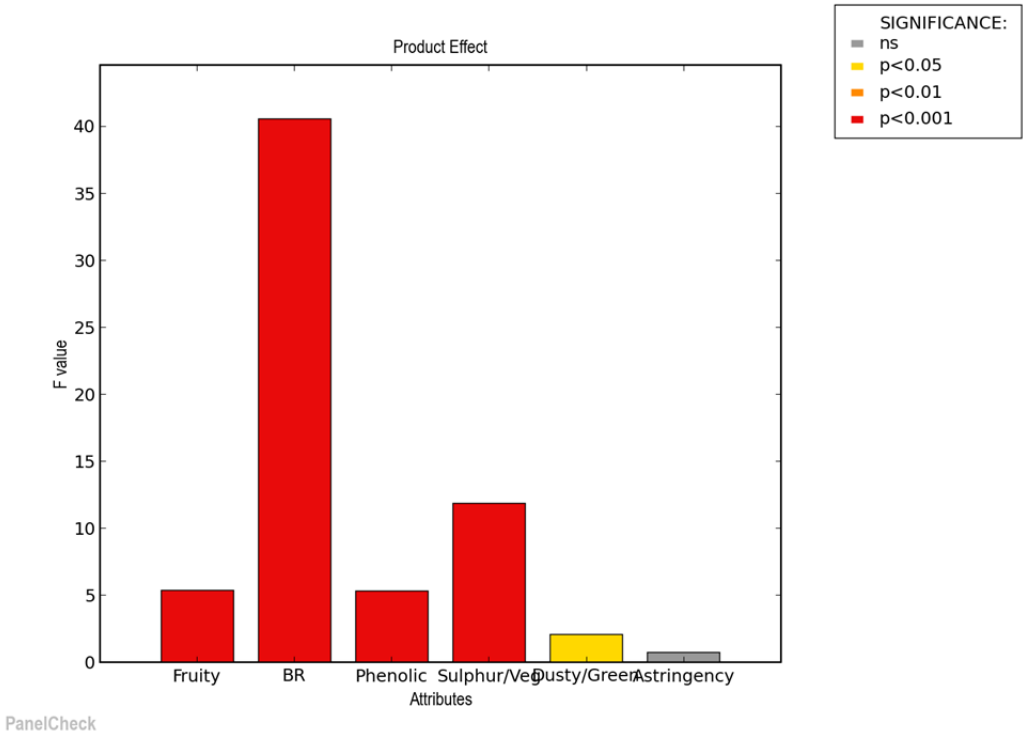


Figure 9: The product effect showing the significant attributes for wines analysed in 2012.

The tucker plot (Figure 8) shows the panellist agreement for fruitiness, burnt rubber (BR), Phenolic and sulphur/vegetative attributes were all significant on a 0.1% level. The burnt rubber attribute had very good agreement amongst panel members, with s F-value of 40. Furthermore the dusty/green attribute were significant on the 5% level, and astringency were not significant in 2012.

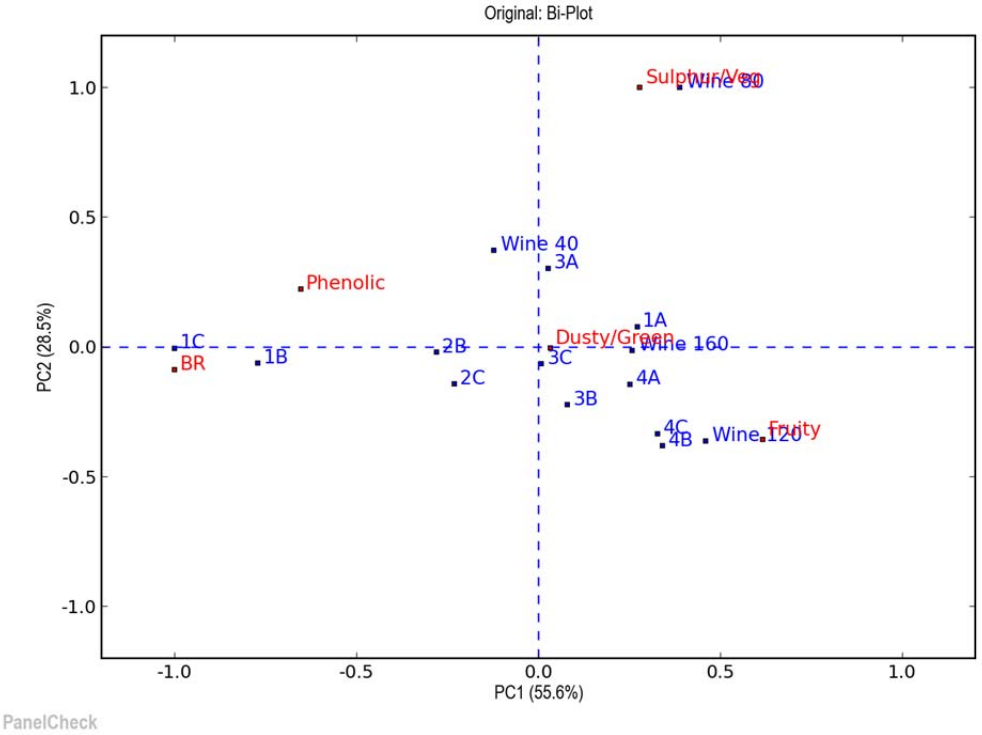


Figure 10: The bi-plot showing the association of all wines made in 2012 with attributes

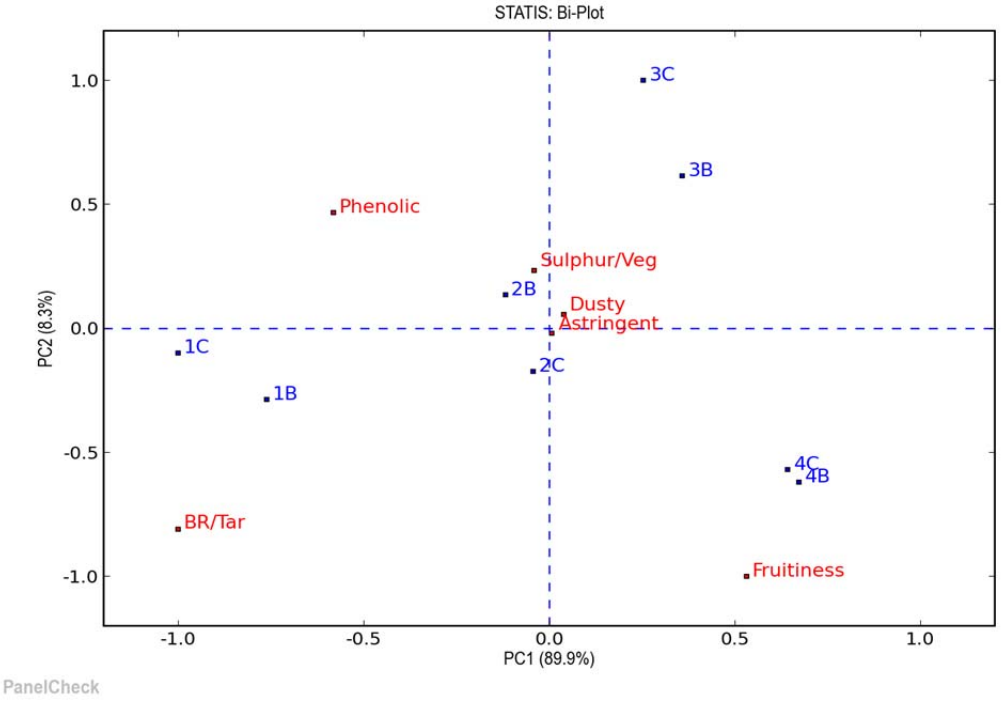


Figure 11: Bi-plot attributes and the association with red wine treatments and blocks

The first PCA (Figure 10) explains 84.1% of the variance within the dataset. Each wine represents the average of the treatments tasted in triplicate. The first principle component, PC1, divides block one and two of the red wine treatments (B and C) from the white, rosé

and block 3 and 4 red wines. The latter are more associated with the fruity attribute; except for white wine from row 80 which was perceived as sulphurous. Furthermore block 1 is associated with the burnt rubber/tar attribute and block 2 shows a weaker association with the burnt rubber and phenolic attributes. The red wines were also plotted in a PCA for further detail. The bi-plot (Figure 11), principle component analysis, shows 98.2% explained variance in the first two principle components for red wines analysed in 2012. The plots show clear distinction between blocks, PC1 splits block 1 and 2 from block 3 and 4. The treatment effect (B and C) does not separating as definitively, indicating small differences between treatments. Block 1 associates with the burnt rubber/tar attribute and block 4 with the fruitiness attribute. Both block 2 and 3 are not associated with any of the significant attributes.

4.3.1.4 Analysis of variance (ANOVA) 2012

In white and rosé wines the burnt rubber/tar attribute was only detected in a single treatment sample (intensity <0.5), and this was not significantly different from the other rows or treatments (intensity = 0). The burnt rubber/tar attribute was associated with pollutants from the stockyard, thus the wines were perceived as clean samples and were further investigated with GC-MS.

The ANOVA least mean square plots of the 2012 sensory data (Figure 12-14) shows the trends per block for the significant attributes (fruitiness, burnt rubber(BR)/tar and phenolic) in red wines. The perceived fruitiness attribute increased as the distance form the stockyard increased; block 1 was significantly lower in fruitiness than block 2, 3 and 4 and block 2 and 3 were significantly lower in fruitiness than block 4 (Figure 12). This shows much clearer effects than seen in 2011, although the intensity of the fruitiness was in the same range for both vintages. The burnt rubber/tar attribute decreased significantly from block 1 to 2, 3 and 4 (Figure 13). The BR/tar attribute was only present in the first two blocks and the intensity was slightly lower in 2012 than for 2011. The phenolic attribute also decrease with increase in distance from stockyard (Figure 14). Block 1 was significantly higher than block 2 and 3, which in turn was significantly higher than block 4.

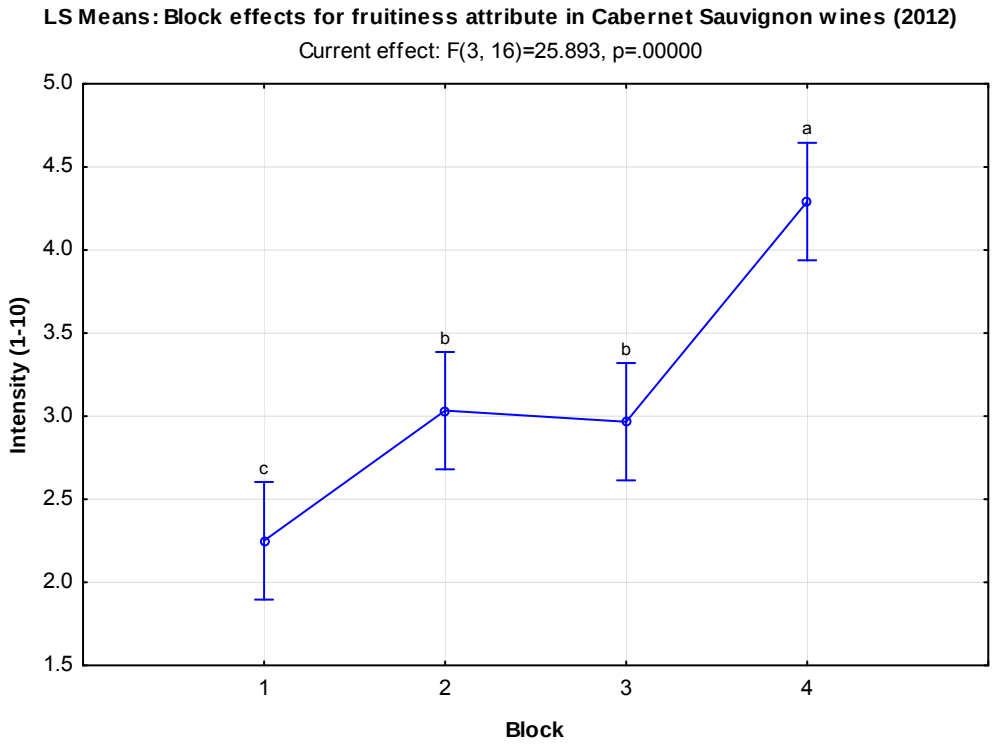


Figure 12: ANOVA Least square means graph showing increase in fruitiness attribute for Cabernet Sauvignon blocks.

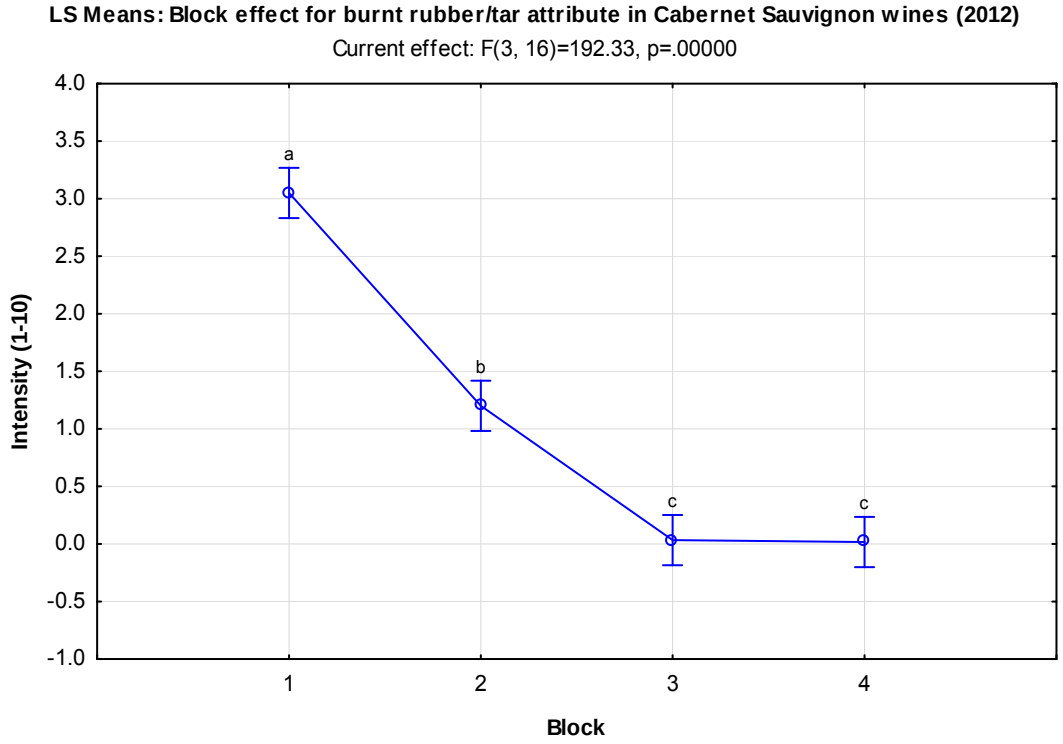


Figure 13: ANOVA least mean square graph showing decrease in burnt rubber attribute for Cabernet Sauvignon blocks. Significant (p value < 0.05) decreases were observed between block 1 and 2, 3 and 4, respectively. There were no significant differences between block 3 and 4.

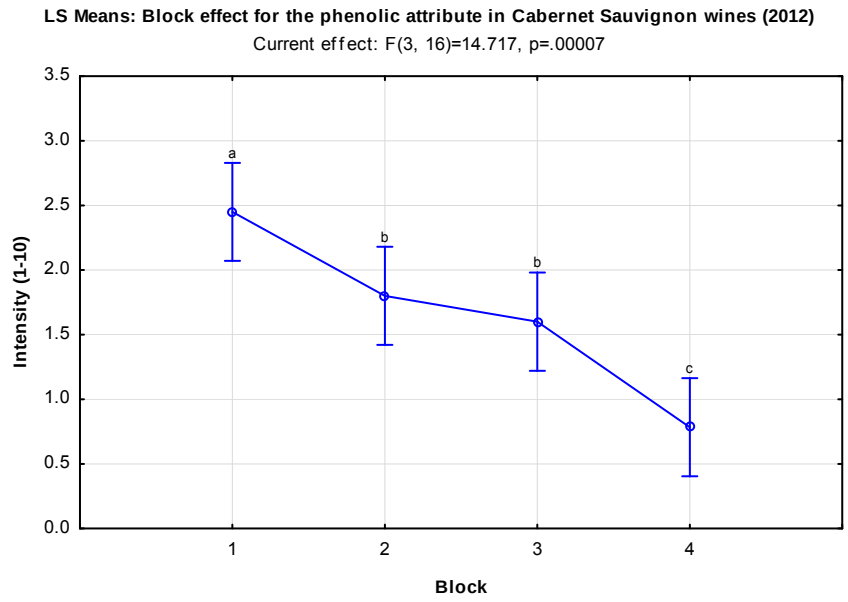


Figure 14: ANOVA least square means graph showing a significant decrease in intensity for the phenolic attribute between block 1 and block 2, 3 and 4 as well as a significant decrease between block 2 and 3, and 4.

From the sensory data obtained, including panel performance analysis, there is a clear difference found between samples. The blocks significantly differed in terms of attributes. Treatment effects between red wines could not be identified sensorially, except for block 1 in 2012. For both vintages, block 1 was associated with the burnt rubber/tar attribute; however the second block's association with this attributes weakened in 2012. This could indicate that the intensity of the perceived burnt rubber/tar attribute found in wines is directly associated with creosote emissions. In this case rehabilitation of vines seems possible. Furthermore there is a definite association between block 4 and the fruity attribute, indicating that the block furthest away from the stockyard is perceived as clean. There was a clear separation between the white and rosé, and the red wines. The red wines were mostly associated with the attributes that contribute to the creosote associated taint in wines, whilst the white and rosé wines were perceived as clean or fruity and grouped away from these attributes. This trend was observed for both vintages, but in 2012 some red wines were also perceived as clean.

Skin contact and fermentation temperature was the main differences between red and white/rosé wine preparation, indicating that these factors may play a role. However, with the 2012 data it is proved to be more of a spatial (block) effect, since some of the red wines grouped with the white and rosé. From the sensory data obtained over two vintages we can conclude that the panel could identify the taints associated with creosote and described it as burnt rubber/ tar. This attribute was found in wine samples from the block 1 and 2 for red wines.

4.3.2 Headspace gas chromatography mass spectrometry

4.3.2.1 White wines (Chardonnay)

The white wines analysed by GC had naphthalene, phenol, 4 ethylphenol, 2,3-xylenol, o-cresol, m-cresol, p-cresol, 4 ethylguaiacol and benzothiophene present. Univariate ANOVA analysis was done on the data to determine the significance of the treatments (row distance from stockyard), vintage effects and the interaction of the treatment and vintage. The vintage had the biggest effect on the concentrations of the compounds found, as illustrated by the naphthalene concentration (Figure 15).

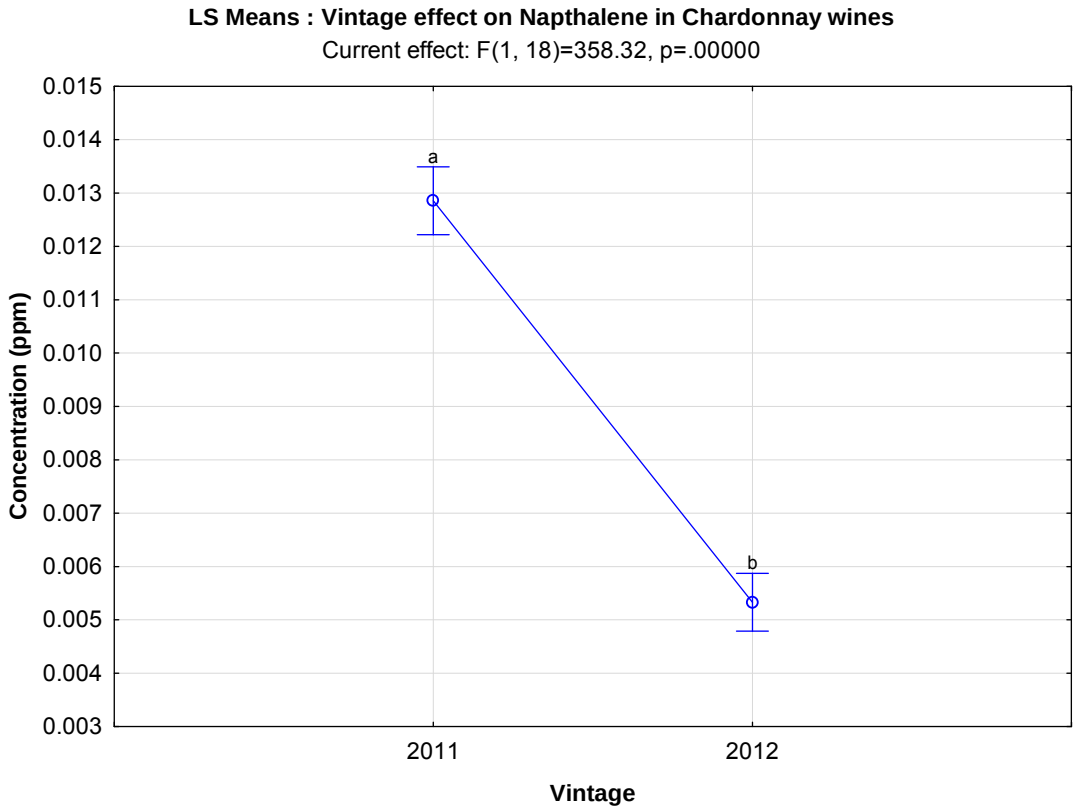


Figure 15: Univariate ANOVA least square mean graph indicating the decrease in naphthalene concentrations from 2011 to 2012.

There was a significant decrease in naphthalene (Figure 15), 4 ethylphenol, o-cresol, m-cresol, p-cresol, benzothiophene and 4 ethylguaiacol concentrations from 2011 to 2012. The decrease in concentrations of these compounds coincided with the closing of the stockyard at the end of 2011. Phenol however had a significant increase from 2011 to 2012. No factors were significant for 2,3-xylenol concentrations.

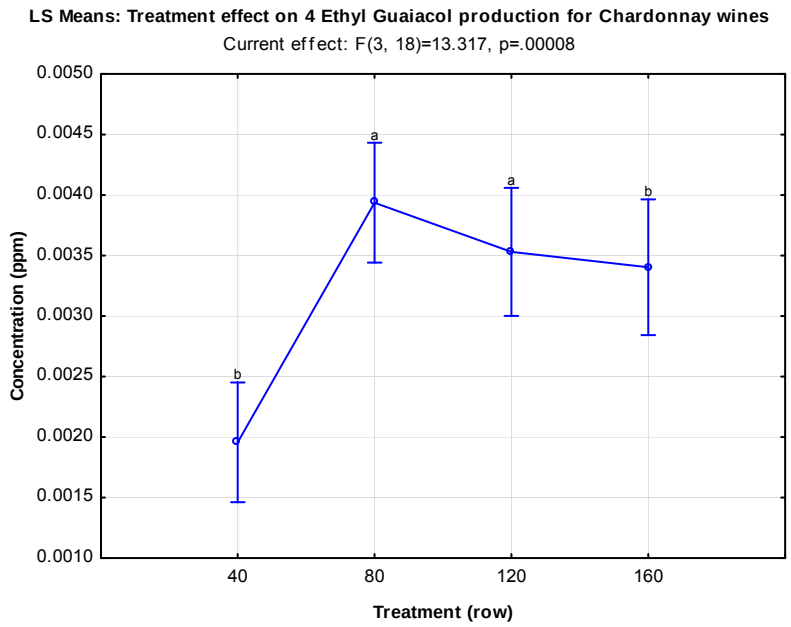


Figure 16: ANOVA least mean plot showing a significant decrease in row 40 from rows 80 and 120.

For only 4 ethylguaiacol the treatment and the year had significant interactions. The treatments, rows, show significant differences between row 40 and 160 and rows 80 and 120. Row 40 has the lowest concentrations and row 80 the highest in 2011 (Figure 16). However, no 4 ethylguaiacol was detected in 2012.

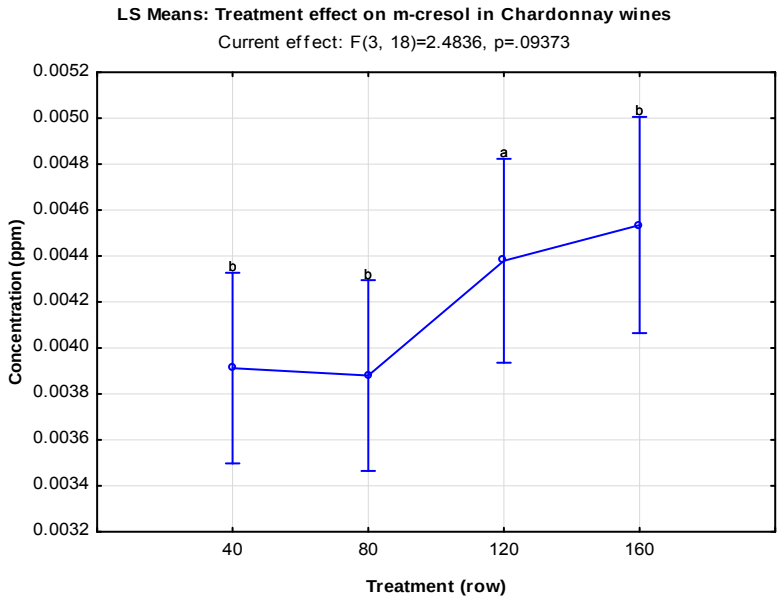


Figure 17: ANOVA Least mean plot indicating an increasing trend in concentration as the distance of the rows to the stockyard decrease.

Trends for naphthalene, m- and p-cresol were observed and all the compounds had an increase in concentrations as the rows increased in proximity to the stockyard - the difference between treatments (row) were however not significant (Figure 17).

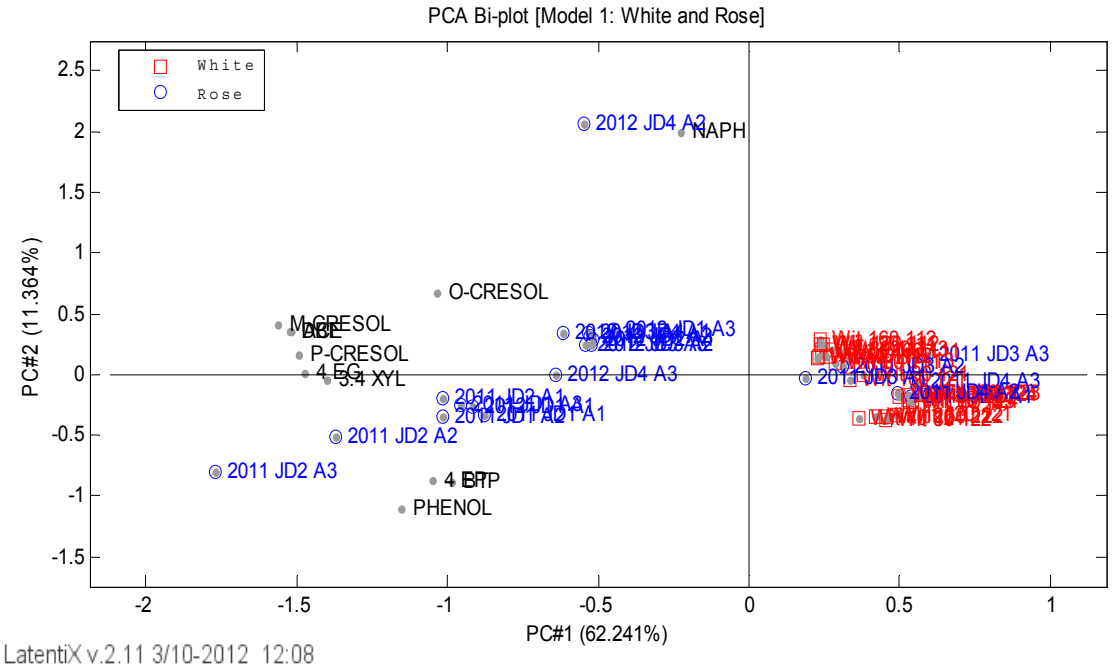


Figure 18: PCA plot showing differentiation between rosé and white wines made from both 2011 and 2012 vintages.

To illustrate the differences in wines perceive as clean during sensory analysis, the GC-MS data of white and rosé wines were compared. The first two principle components explain 73.6% of the variance within the data set (Figure 18). PC1 does however separate some rosé wines from all the white wines. There is a clear association with some of the rosé wines with the VOCs, whilst there was no association from the white wines. There were no vintage or block significance for the rosé wines that associated with the compounds.

4.3.2.2 Red wines (Cabernet Sauvignon)

Compounds found in red wines were naphthalene, phenol, 4 ethylphenol, 3,4-xylenol, 2,3-xylenol, o-, m- and p-cresol, acenaphthene, dibenzofuran, 4 ethylguaiacol and benzothiophene were detected in the red wine samples. Univariate ANOVA analysis was done with STATISTICA to determine the effect of vintage, block (1-4) and treatment (A-D) as well as the interaction between these factors on each compound found in red wine. This was done to determine the biggest influence for the concentrations of the compounds detected in wine. The year, block and treatment effects were significant, p value <0.05, for phenol, 4 ethylphenol, 3,4-xylenol, o-cresol, m-cresol, 4 ethylguaiacol, acenaphthene and 2,3-xylenol. For all compounds the concentrations found in wine decreased from block 1 to block 4. Treatment C had the highest concentrations of compounds, followed by treatment B

and D, and lastly A, this trend however varied according to the compound of interest. Only naphthalene was found to be non-significant, this could be due to the presence of outliers within the data set, or the volatile nature of the compound.

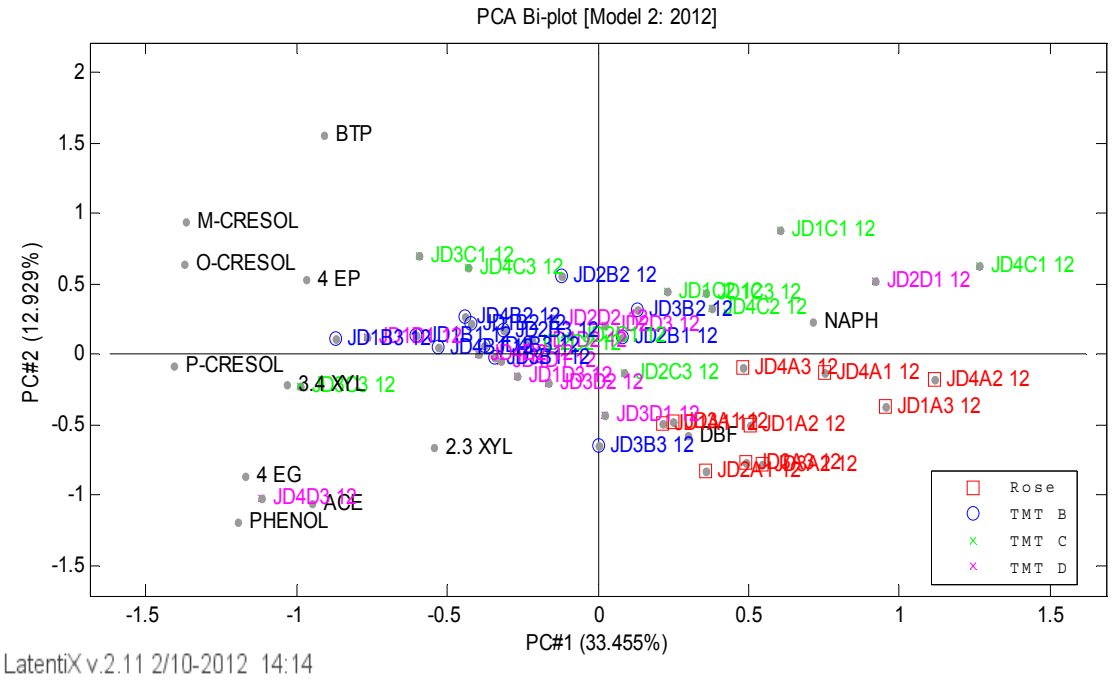


Figure 19: PCA plot of VOCs associated with different treatments of Cabernet Sauvignon wines.

The first two principle components illustrate only 46.4 % of the explained variance within the dataset (Figure 19). All the red wine treatments from 2012 are plotted with the VOCs measured by GC-MS. Although the explained variance is not good enough to make concrete conclusions, a general trend can be observed. All the rosé wines, treatment A, is separated from the compounds of interest by PC1, whilst the red wine treatments, B,C and D is more associated with the compounds, in particular treatment B.

Analysis of variance (ANOVA) for Cabernet Sauvignon wines

The one-way ANOVA analysis was done on the concentrations of VOCs found by GC-MS method with a 5% LSD test. In red wines there were more significant interactions of compounds and the compounds that had important effects were also different from white wines. Compounds concentrations generally decreased from block 1 to block 4, but showed an increase from 2011 to 2012. The 2012 season was longer, and grapes were harvested two weeks later than 2011; this led to a prolonged exposure of grapes to VOCs emitted and may explain the higher concentrations in 2012. Alternatively this could also be due to the analysis that was done in 2012, which could have led to a decrease/degradation of volatile compounds in 2011 wines. This trend was not observed for all compounds. In 2011, block 1

and 2 had much higher concentrations of phenol than block 3 and 4 for treatments A and C. In 2012, however, less differences between blocks and treatments could be observed, with the only significant difference was treatment C, block 3, was significantly different from block 1, 2 and 4 (Figure 20). There were more significant differences between treatments and blocks in 2011 versus 2012 for all the compounds measured, although 2012 did not show a significant decrease in compounds as was shown in white wines.

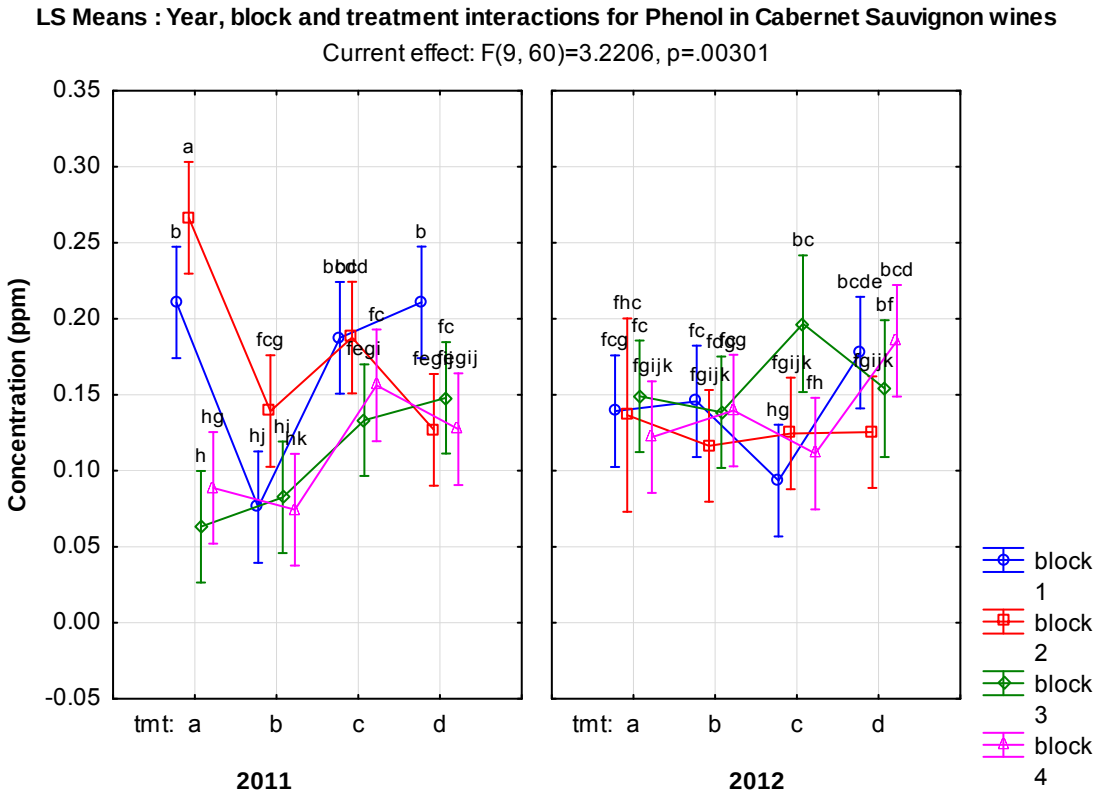


Figure 20: Univariate ANOVA LS means graph of vintage, block and treatments effect influencing Phenol concentrations in red wine.

In 2011 the phenol concentrations were significantly higher for treatment A, B, and D for block 1 and 2. Figure 20 shows that these blocks had the highest concentration of phenol in 2011, in 2012 no significant differences were observed. The phenol concentrations of the rosé wines (treatment A) had higher concentrations than the other treatments in 2011. This indicates that phenol is not extracted during skin contact procedures, but rather is concentrated in the berry. From sensory analysis a decrease in the burnt rubber attribute for all rosé and white wines were observed, and thus this compound is probably not a major role player in the taint, but may act synergistically with other compounds. During further investigation phenol showed contribution to sweet and fruity attributes and were not associated with the burnt rubber taint (Panzeri, 2012).

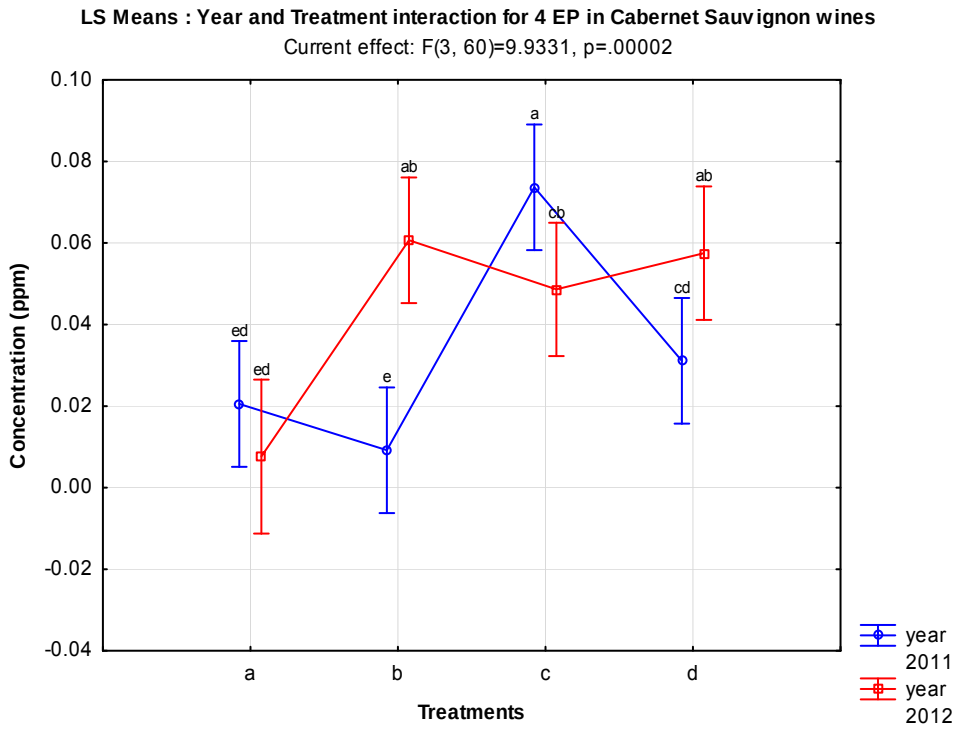


Figure 21: Univariate ANOVA LS means analysis shows significant differences between treatments and year for 4 Ethylphenol (4EP) concentrations (ppm) measured in Cabernet Sauvignon wines.

One of the defining compound behaviours in red wine was 4 ethylphenol, usually associated with *Brettanomyces sp*. infections in wine, this compound is also a volatile constituent of creosote emissions. For 2012 the concentrations found were significantly lower for treatment A (Figure 21), indicating that this compound may be extracted from the skins. This was however not the case in 2011, where only treatment C was significantly higher in 4 ethylphenol. The 2011 wines were only analysed in 2012, which might have had an effect on the concentrations of this compound. When only the block effect is taken into consideration, with an average of the other factors, a decrease can be observed from block 1 to block 4 (Figure 22). This can give insight as to the distance the compound can spread in the atmosphere as block 1 is significantly higher than block 3 and 4. The highest average per block was 0.05 ppm, which is 500 ppb, just above the detection threshold (Table 3) in wine; the other 3 blocks however was below threshold. The concentration of 4 ethylphenol found is most likely to have arisen from the volatile emissions of the stockyard due to *Brettanomyces sp.* preventative and controlled winemaking.

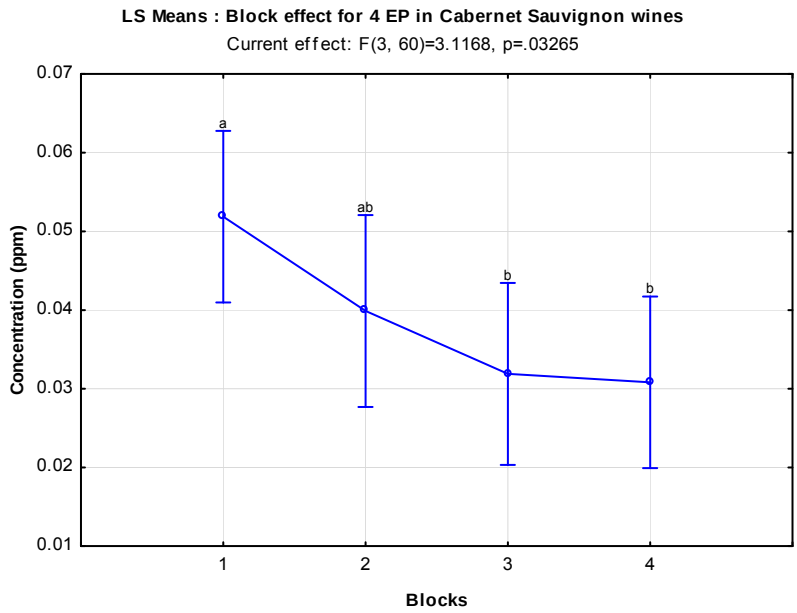


Figure 22: Block effect for 4 ethylphenol, this trend was observed for most compounds. Significant difference ($p<0.05$) can be seen between block 1 and 3 and 4, block 2 however, does not significantly differ from either blocks.

Another compound that had interactions between all the factors measured were 4 ethylguaiacol, the compound usually associated with smoke from bushfires. Similar to 4 ethylphenol there were significant differences only in 2011 between block 1 and 2 and block 3 and 4 for treatment A, only block 2 was significantly higher for treatment B, block 1 was significantly higher than the other blocks for treatment C and there were no significant differences between the blocks for treatment D (Figure 23). In 2011 treatment C for block 1 was significantly higher than the other blocks and treatments, except treatment A for block 2, this compound is thus not influenced by water washed grapes. For the vintage and block interaction there were no significant differences in 2012, but for 2011 block 1 and 2 were significantly higher than block 3 and 4.

Although there was a fire in the surrounding mountains in 2011, the effect would not have contributed to a block effect, as all the blocks would have been equally exposed due to the location of the fire being parallel to the four blocks. It could however have played a role in the vintage effect; however concentrations in 2012 were comparable to 2011.

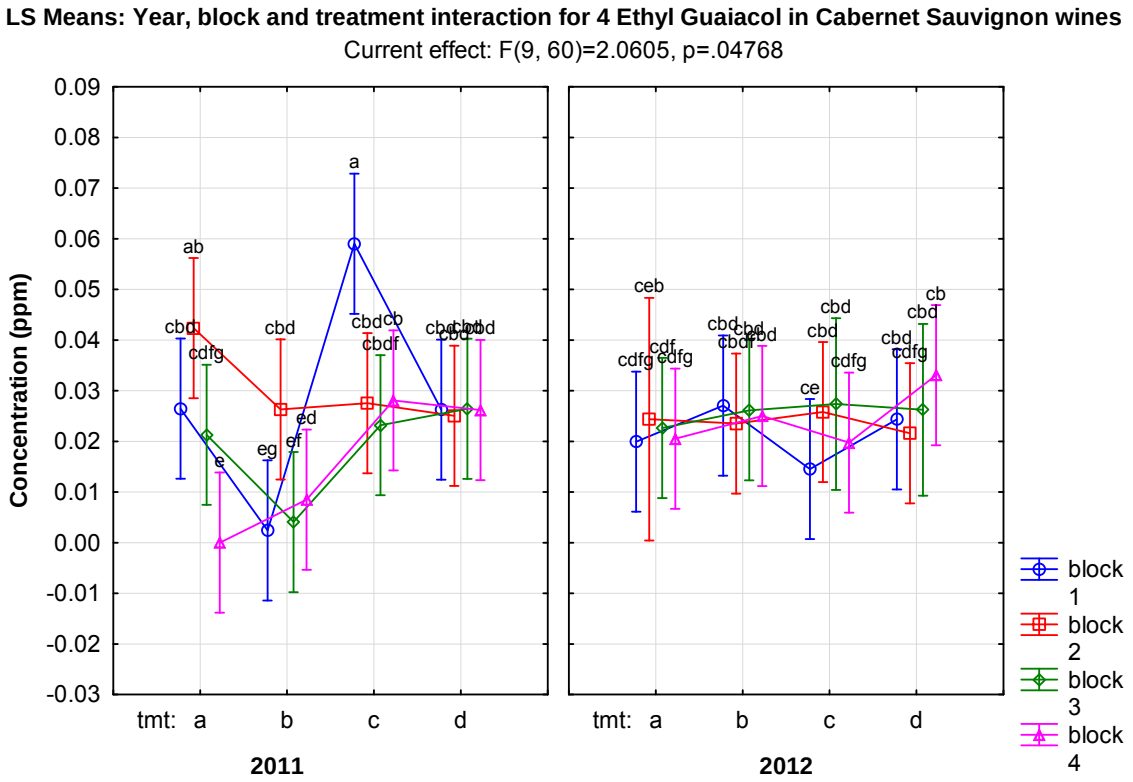


Figure 23: Effect of year, block and treatment interaction on 4 Ethylguaiacol concentration found in Cabernet Sauvignon wines made from two vintages.

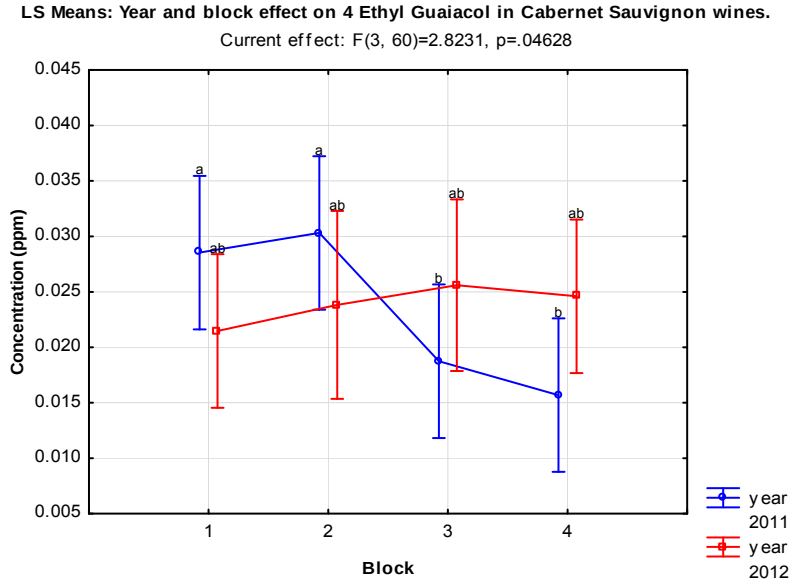


Figure 24: The year and block interaction on 4 Ethylguaiacol concentrations found in Cabernet Sauvignon wines.

From literature we have comparative concentrations of volatile cresols and phenols from chemical and sensorial analysis in wine and water. A recent sensorial study on odour identification found o-cresol, m-cresol and p-cresol at 62, 20, and 64 ppb, respectively in smoke effected wines. In wine that was made under similar experimental conditions , with no

malolactic fermentation, red wine, Cabernet Sauvignon, showed odour detection thresholds of 9, 4 and 8 ppb for o-, p- and m-cresol respectively (Parker, *et al.*, 2012). The concentrations found in this study were higher than found by Parker, *et al.*, in 2012, but only m-cresol was present in red wines above the detection threshold of 20 ppb. Furthermore m-cresol has a tarry, smoky, medicinal odour (Table 3), and can therefore be linked to the burnt-rubber attribute that was described sensorially. This compound was the only compound above literature odour thresholds, but due to the complexity of the taint found in wine and creosote emissions, other compounds may contribute that were not analysed for at the present time. These include styrene, toluene and benzene, amongst other compounds. In white wine however, the rows were not as significantly different, indicating that this block may have not been influenced by the stockyard as much as the directly exposed Cabernet Sauvignon block was. Furthermore, naphthalene, o-, m- and p-cresol, phenol, 4 ethylphenol and 4 ethylguaiacol showed strong associations with blocks, indicating that these volatile compounds most likely spread via the atmosphere and contaminated the vines.

Table 5: Summary of GC-MS results for wine, berries and leaves.

Compounds	Red Wines			White Wines			Berries			Leaves			OT in wine
	total	σ	Average(ppb)	total	σ	Average(ppb)	total	σ	Average(ppb)	total	σ	Average(ppb)	ppb
Naphthalene	0.7796	0.0080	8.4736	0.2213	0.0039	8.5111	2.2266	0.0794	35.3433	0.0848	0.0083	5.3005	**84**
Phenol	12.9789	0.0519	141.0747	2.9593	0.0347	113.8191	145.0250	9.9343	2301.9843	57.0055	7.4310	3562.8462	**2000-4000**
4 Ethylphenol	3.6051	0.0368	39.1862	0.0892	0.0048	3.4322	16.3230	0.9189	259.0949	5.3276	0.5102	332.9737	**300**
3,4 Xylenol	8.3049	0.0302	90.2704	1.0209	0.0113	39.2654	nd	nd	nd	nd	nd	nd	
o-cresol	2.8878	0.0112	31.3893	0.3224	0.0134	12.3989	313.3647	22.5708	4974.0434	124.4090	20.6892	7775.5610	**63**
p-cresol	2.5889	0.0176	28.1398	0.1124	0.0052	4.3220	1.1588	0.0545	18.3937	3.4424	0.4909	215.1505	**64**
m-cresol	2.2146	0.0106	24.0721	0.0912	0.0042	3.5066	13.2564	0.7857	210.4194	11.1436	1.3880	696.4760	**20**
4 Ethylguaiacol	2.1627	0.0146	23.5074	0.0702	0.0034	2.6983	1.7988	0.0503	28.5521	nd	nd	nd	**50**
Acenaphthene	1.3230	0.0073	14.3799	nd	nd	nd	0.4235	0.0045	6.7227	0.0300	0.0029	1.8759	
Benzothiophene	1.1312	0.0067	12.2954	0.0714	0.0022	2.7465	0.5304	0.0134	8.4196	0.0984	0.0031	6.1510	
Dibenzofuran	0.9391	0.0058	10.2080	nd	nd	nd	0.4295	0.0068	6.8179	0.0635	0.0036	3.9689	
2,3 Xylenol	0.4402	0.0144	4.7847	0.0302	0.0024	1.1616	3.9125	0.3258	62.1029	0.5136	0.0636	32.1001	
2,6 Xylenol	nd	nd	nd	nd	nd	nd	0.6520	0.0241	10.3499	nd	nd	nd	

4.3.2.3 Berries (Cabernet Sauvignon)

The berries were analysed with SPME HS GC-MS to determine the effects of factors such as year, sampling date, skin vs. pulp contamination, block effect and the interaction between these factors. The compounds that were measured in the berries are naphthalene, phenol, 4 ethylphenol, 2,3-xylenol, o-cresol, m-cresol, p-cresol, dibenzofuran, acenaphthene,

benzothiophene, 4 ethylguaiacol and 2,6-xylenol. Statistical analysis of each compound and the factors influencing the concentration (ppm) was carried out. General trends were observed for most compounds. These were:

- concentrations were higher during the second sampling date (higher sugar and thicker cuticle wax layer),
- higher concentrations in the skins vs. the pulp and a decrease from block 2 to block 4 in concentrations (Figure 27).
- The concentrations were lower for 2012, than for 2011.

Few compounds however showed significant interactions between the factors influencing the concentrations, 2,6-xylenol, 4 ethylguaiacol, acenaphthene, dibenzofuran, m-cresol and naphthalene had interaction between factors however. Some of these results will be discussed briefly.

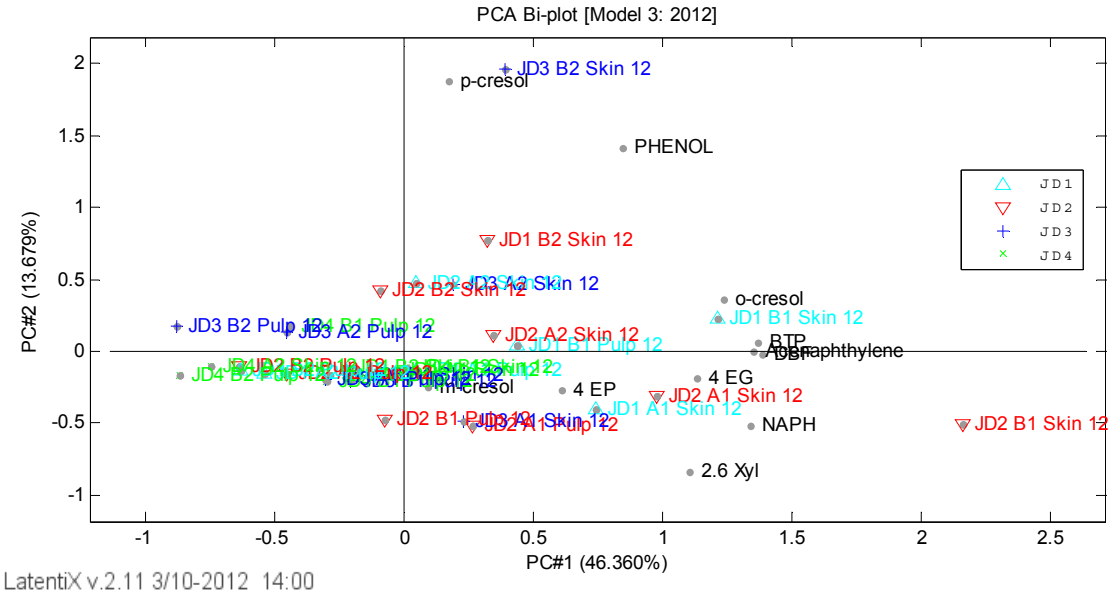


Figure 25: Principle Component Analysis on berries according to block.

The first two principle components explain 60% of the variance within the sample set (Figure 25). Although the explained variance is relatively low, a trend can be observed. The PCA plot shows the higher correlation of block 1 and 2 with the compounds associated with the volatile fraction of creosote. The variance within the system may have been improved with more replicates included in the sampling and by the removal of outlier samples.

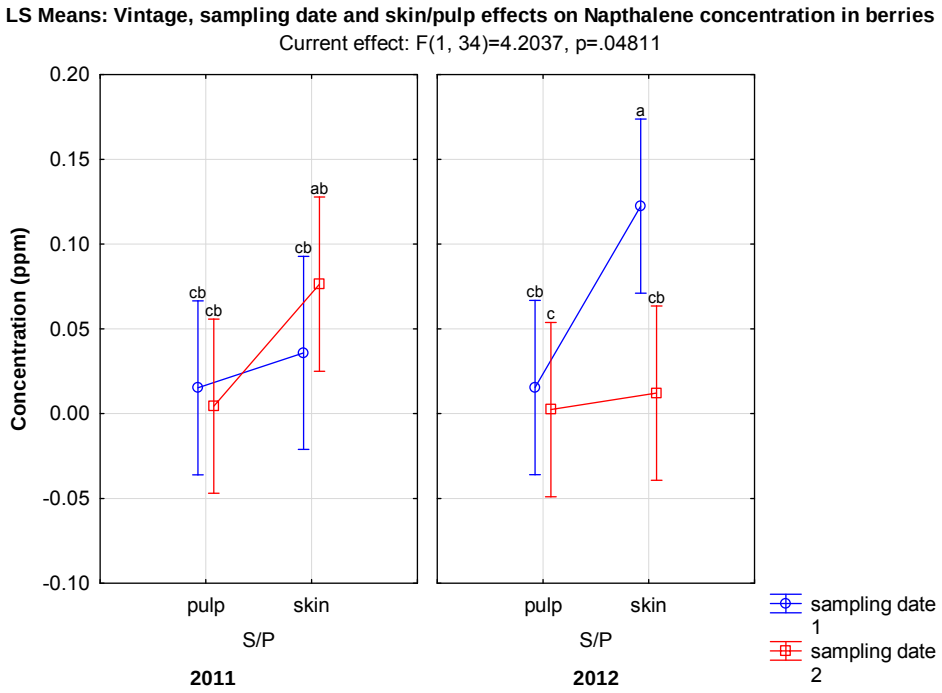


Figure 26: Naphthalene concentration (ppm) in berries as influenced over two vintages, skin and pulp and sampling dates.

No significant differences for naphthalene were observed between skin and pulp in 2011 (Figure 26), however for the first sampling date in 2012 the skin's concentration were much higher than concentrations present in the pulp. By the second sampling date this effect was no longer significant and the skin only has a slightly higher concentration than the pulp, as is seen in 2011 (Figure 26). Block effect of Naphthalene (ppm) shows a significant (5% confidence level) decrease in concentration from block 2 to block 3 and 4 (Figure 27), indicating highest concentration in block 1 and 2 closest to the stockyard. Both acenaphthene (Figure 28) and dibenzofuran showed different trends for the sampling dates. All the other compounds, except 2, 6-xylenol, had higher concentrations in the second sampling date, regardless of the vintage. Both these compounds however showed an increase over time in 2011, whilst in 2012 it showed a decrease with the second harvesting date. The only significant differences between these concentrations were between the second sampling date in 2011 and 2012.

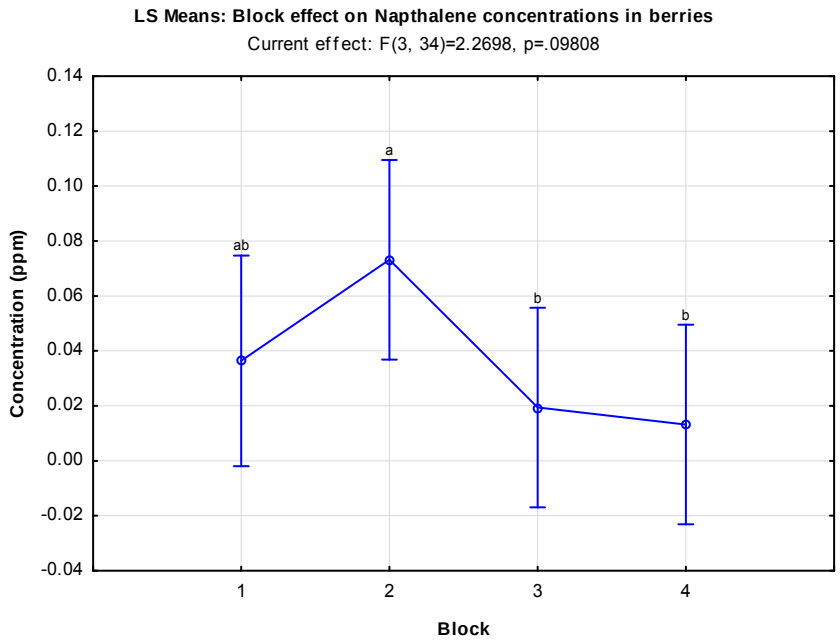


Figure 27: Block effect of Naphthalene concentration (ppm) measured in Cabernet Sauvignon wines made from block 1 to 4.

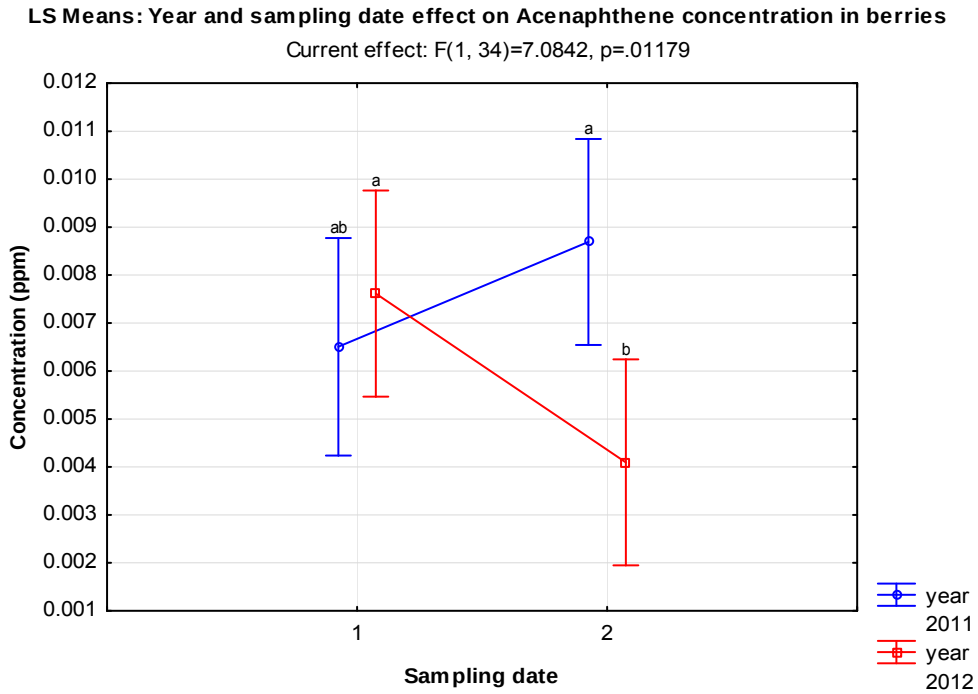


Figure 28: Acenaphthylene (ppm) concentrations as influenced by year and sampling date

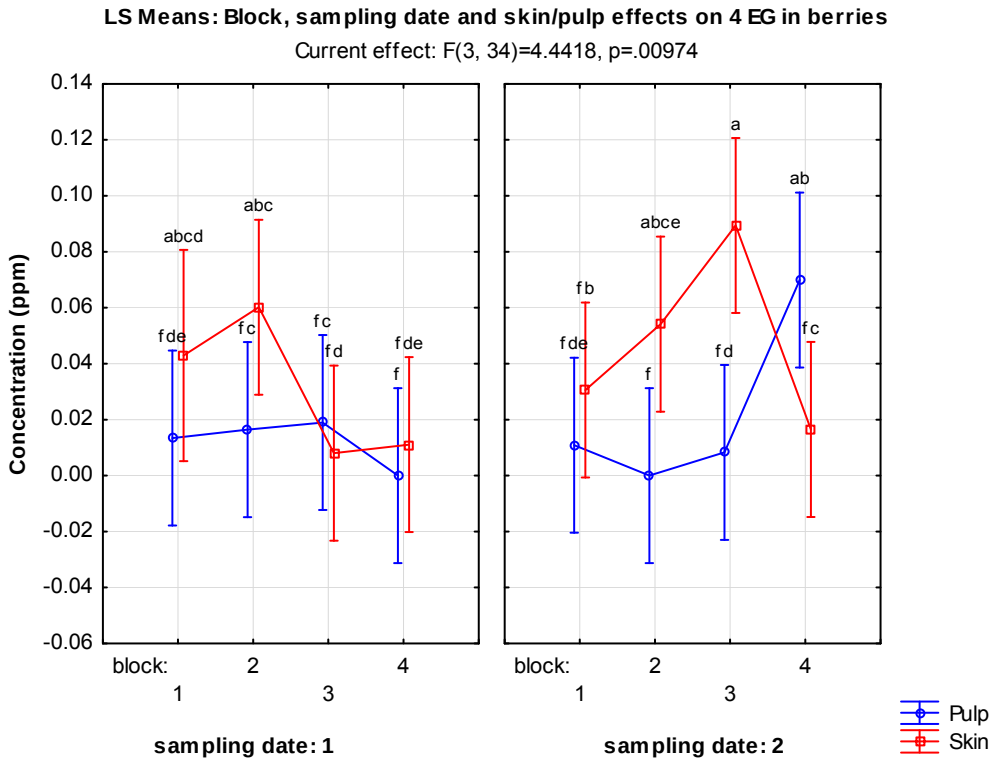


Figure 29: Least Squares plot for the interaction between block, sampling date, skin and pulp effects on 4 Ethylguaiacol concentrations in ppm.

An interaction between block, year and skin/pulp could be seen for 4 ethylguaiacol. During both vintages the skins had a higher concentration than the pulp, although these effects were only significant in 2012 for the 2, 3 and 4 block (Figure 29). During 2011 there were a general decrease in 4 ethylguaiacol concentrations, in 2012 there were a significant increase seen in skins and pulp.

Concentrations of the volatile phenolic compounds found in berries were higher than for wines (Table 5). Acenaphthene and dibenzofuran were measured in berries but not detected in white wine, whilst 2,6-xylenol were detected in berries and not detected in red or white wines. These compounds may thus be lost during fermentation, either by volatilization or binding to particulates formed during fermentation. Phenol, o-cresol and m-cresol were found at concentrations higher than the odour threshold in wine (Table 5). For these compounds there were no significant effects but all compounds showed higher concentrations for the second sampling date, skins and in year 2011. Therefore these compounds are associated with the emissions from the stockyard (decrease in concentrations in 2012) and accumulate in the skins over the ripening season. The compounds are not all transferred to wine at detectable odour levels.

4.3.2.4 Leaves

Leaves were analysed by SPME HS GC-MS method for volatile phenols. The compounds found in the leaves were naphthalene, phenol, o-cresol, m-cresol, p-cresol, 2, 3-xylenol, 4 ethylphenol, benzothiophene, acenaphthene and dibenzofuran. Factors measured to influence the presence of these compounds included the block were the leaves were sampled, as well as year.

The leaves showed no significant trends between the blocks and the vintages. However, the volatile phenols measured in the leaves were at higher concentrations than found in wines and berries. Phenol, 4 ethylguaiacol, o-, m- and p-cresol was present at higher concentrations than the odour threshold in wine (Table 5). The levels found for 4 ethylguaiacol, m-cresol and p-cresol were also higher than the odour threshold for these compounds in air (Table 3). The volatile compounds may accumulate on the leaves at higher concentrations than the berries due to the bigger exposed leaf area, as was found by other studies (Kipopoulou, *et al.*, 1999; Wang, *et al.*, 2010). A further indication of pollution from the stockyard would be to measure the air contamination, by means of glass air traps and the analysis of leaves for PAHs, as PAHs area lipophilic and may accumulate on the leaf wax layer.

4.3.3 High performance liquid chromatography-diode array detector

Only Chrysene was found in red and white wine treatments made from exposed blocks. Univariate ANOVA analysis was done with STATISTICA on the HPLC data.

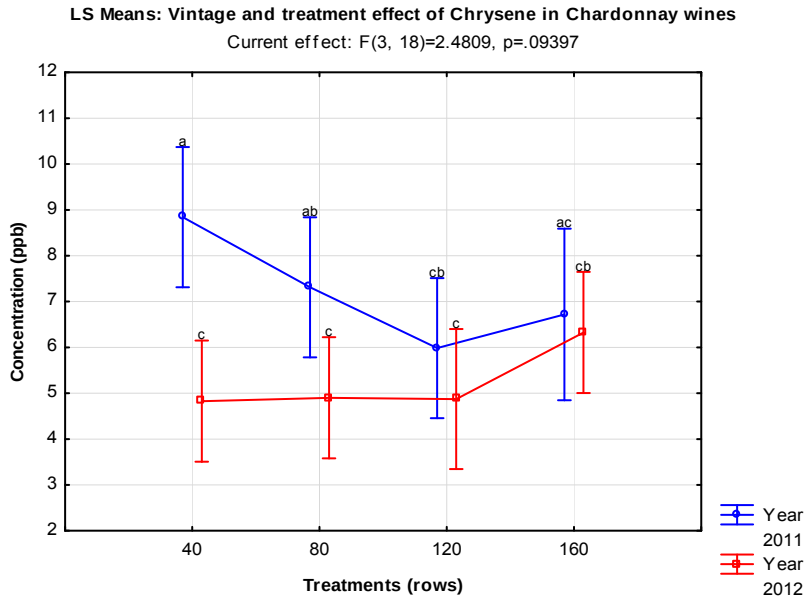


Figure 30: White wine HPLC PAHs analysis for chrysene (ppb) measuring year and treatment effects.

Of the factors influencing the concentration of chrysene found in white wines, only the vintage was significant on a 5% level (Figure 30). There was a significant decrease in chrysene concentration from 2011 to 2012. Neither the treatments nor the interaction of vintage and the treatments were significant, however a trend can be observed in Figure 30. The treatments were different rows, 160 being the closest and 40 the furthest from the stockyard. For each row the concentration of chrysene present in the wines were higher in 2011 than 2012, but only row 40 and 80 showed this trend significantly on a 5% level. In 2011, only row 120 was significantly different, but only from row 40. Row 40 had the highest concentrations of chrysene and being furthest from the creosote stockyard, the contribution of creosote emission to PAH concentration is highly unlikely, especially considering chrysene is non-volatile. Other contributing sources of PAHs include, factory emissions and vehicle emissions, the vineyard would most likely be polluted with vehicle emissions during viticultural practices or from other farm activities, since the factory source of pollution is further than the stockyard.

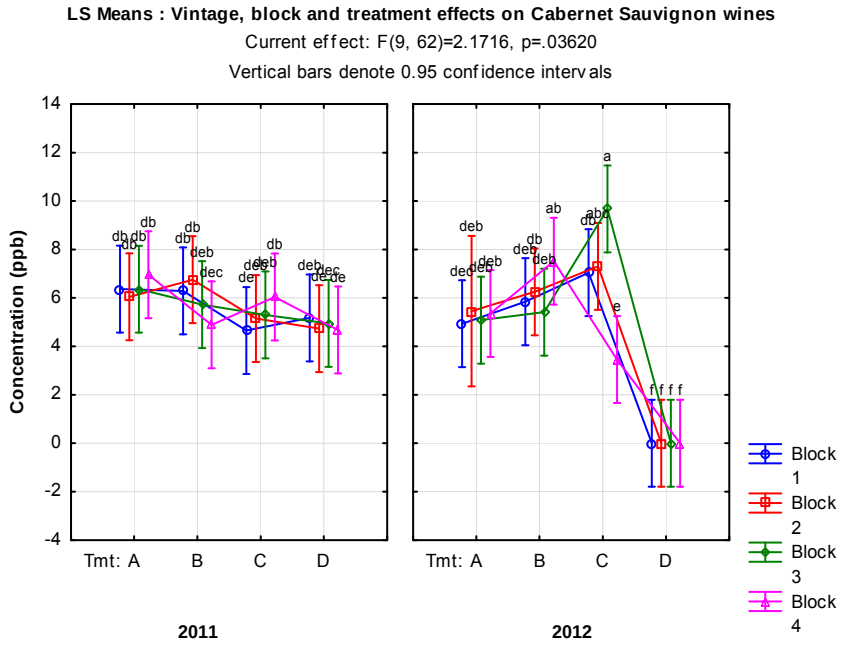


Figure 31: Interaction of the LS means of chrysene according to treatment, block and year on the Cabernet Sauvignon red wines made from blocks adjacent to the Cabernet Sauvignon blocks.

In year 2011, when the stockyard was still open, all the red wines treatments had chrysene present at a concentration of 5 to 7 ppb. European studies conducted by European Food Safety Authority in 2008, noted that in samples where benzo(a)pyrene was not detected, chrysene was found in concentrations up to 242 ppb. However in 2012 there were significant differences between treatments and blocks present (Figure 31). Block 3 and 4 are significantly different for treatment C from block 1 and 2, where block 3 has the highest

concentrations found, 10 ppb. Furthermore, all the blocks were significantly different on a 5% level ($p<0.05$) for treatment D in 2012, which is expected as the waxy layer was removed with ACN, and could remove the lipophilic PAHs (Figure 31).

The concentrations found in red wines were much higher than found in literature, (0.4 ppb), by Chatonnet & Escorbessa in 2007. Thus the environmental impact in terms of PAHs pollution of the creosote plant has contributed to a very high concentration of PAHs for wines. The legal limits for food range from 1-10 ppb, from infant food to bivalve molluscs, respectively. Only the Czech Republic has legislation for wine and that is limited to 0.5 ppb for the total of 8 PAHs, which includes chrysene (Wenzl,*et al.*, 2006). The wines have 10 times higher concentration for only chrysene. This was observed more for red wines, and thus these wines can be seen as a human threat and should not be available to the market until rehabilitation of the area has been done. The red wines show the highest concentrations of VOC measure as creosote related compounds. There were significant differences in terms of block, treatment and vintages for most of the compounds detected. In red wines, a clear trend was observed for all wines showing the decrease in VOC concentration as the distance of the block from the source of contamination decreased, however the PAHs were omnipresent in all the wines.

4.4 CONCLUSION

The health of a vineyard has an important influence on the quality of wines produced. Not only are the environmental practices applied important for the quality of the fruits obtained, it also has become increasingly important to the consumers of these products. Food safety legislation is therefore set up in countries to ensure that what is produced is safe for consumption and ensures sustainability for the industry. The research done on wines, berries and leaves in terms of VOC and PAHs from a creosote source, aims to better understand the influence there of, comparable to any industrial activity, in close proximity to the growing area. It is well documented that creosote has negative effects on the environment and is responsible for toxic pollutants in secondary products. From this study it is evident that creosote has an important effect on wine, as volatiles deposited on the grapes survive the winemaking process and are sensorially perceived as taint. We were able to determine the identity and quantify some of these compounds using HPLC and GC-MS, and showed that the PAH concentrations were alarmingly high compared to legal limits in literature. Sensorial differences between blocks showed a descending trend in the associated taint as distance from the source increased along with an increase in fruity character. The VOC and sensorial data did not correlate in trends between treatments and blocks; this could be due to the individual behaviour of the taint compounds in terms of their

deposition, uptake and transfer patterns. This bears further investigation. The inclusion of methyl styrene, indene, biphenyl, quinoline and isoquinoline in such a future study could further help to explain the sensorial taint present.

Further research could thus be done on the PAHs uptake of the vine, transportation of VOC and PAHs and the environmental impact of industrial activities within an agricultural area. Recommendations include the revision of creosote use in agriculture, especially for viticulture, and the bio-remediation practices of contaminated areas to ensure sustainable practices and low levels of PAHs and VOCs in wines.

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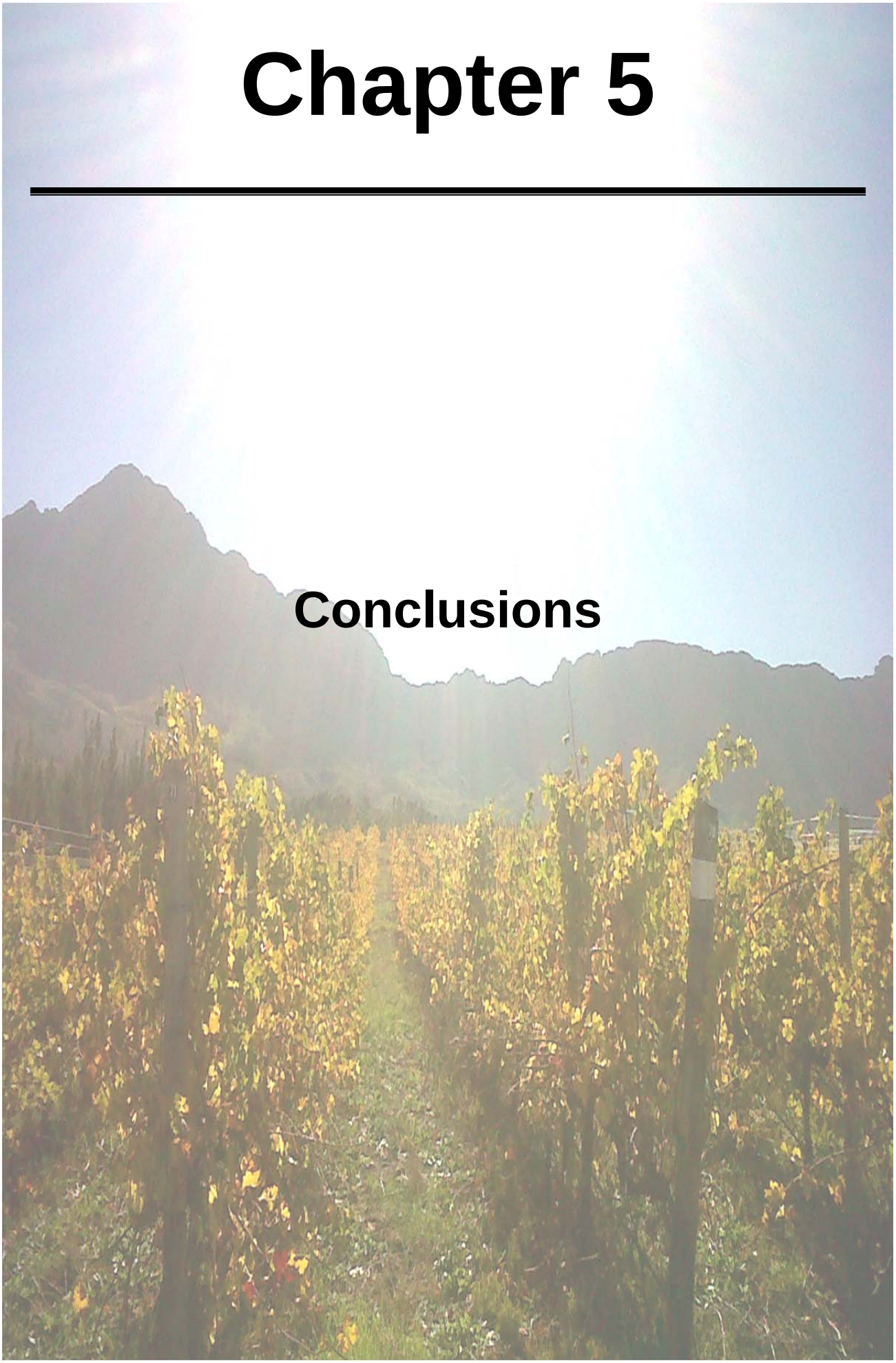
Chapter 5



Conclusions

5.1 CONCLUSIONS

Creosote is a product of crude tar distillation and is applied for the preservation of wood. Poles treated with creosote are used for various industries including railway-ties, telephone-poles, built environment structures and trellising systems in the agricultural sector (World Health Organization (WHO), 2004). Creosote contains carcinogens due to its 85% PAH content, and other constituents include phenolic compounds, N-, S- and O- containing heterocycles (Meyer, *et al.*, 1999). Creosote usages have been restricted in the European Union, United States of America, Canada and Australia, but in South Africa no restriction as of yet exists for creosote usage as trellising stakes in agriculture (Mateus, *et al.*, 2008), although farmers are required to use only posts that have been treated according the SABS 457 standard.

Various studies on the toxic effects of creosote on air, soil, water and plant health have been carried out (Neubauer & Kasimatis, 1966; Hale & Aneiro, 1997; Meyer, *et al.*, 1999; Eriksson, *et al.*, 2001; Moret, *et al.*, 2007; Mateus, *et al.*, 2008). The health of the vines, wines and environment is a major concern, especially in terms of international trade and sustainable farming. The decrease of quality of wines directly associated with creosote constituents and the sustainability of the holistic environment are also issues associated with the use of this preservative.

No literature was found on vines or wines being monitored for creosote pollutants. However information of PAHs in foodstuffs and drinking water is available as the United States, and most EU countries have strict legal limits for PAHs (Wenzl, *et al*., 2006). PAHs are now monitored in food, water and air and sixteen compounds, mostly higher molecular weight PAHs, have been prioritized due to carcinogenic and mutagenic properties. Benzo(a)pyrene is considered a marker molecule for PAHs contamination (Environmental Protection Agency, 1985) . According to legislation creosote must contain less than 50 ppm of benzo(a)pyrene in mass, drinking waters must contain 0.1ppb of total PAHs. Foodstuff legislations vary according to country but in general range between 0.1 to 10 ppb (Commission Regulation (EC), no 208, 2005). Only Czech Republic has legislation of PAHs in wines and a legal limit of 0.5 ppb has been set (Wenzel, 2006).

Studies for PAHs in wines investigated toasted barrels as the main source of these compounds. All the studies conducted found concentrations below 0.5 ppb, and concluded that using toasted barrels as part of oenological practice would not contribute to PAHs associated with health risks (Moret, *et al.*, 1995; Chatonnet & Dubourdieu, 1998; García-Falcón & Simal Gándara, 2005; Chatonnet & Escobessa, 2007).

This study aimed to determine the environmental effect of a creosote stockyard on a commercial wine farm. Samples that were analysed included soil, water, grape berries,

leaves and wines made from blocks adjacent to the stockyard. Chapter 3 focuses on the environmental impact of the creosote plant, in terms of PAHs content of river water and soil as well as volatile organic compounds present in the air and available for plant uptake via the leaves (aim i & ii). Chapter 4 mainly focussed on the vineyard affected by the creosote contaminants in terms of grape and wine volatile phenol and PAH content (aim iii & iv). Various analytical methods had to be developed for the analysis of these samples. Headspace gas chromatography mass spectrometry and high performance liquid chromatography methods were developed for the analysis of VOC and PAHs, respectively (aim i). Sample preparation methods were developed for wine, and other matrices were adapted from existing methods. The aims, as set out in Chapter 1, were achieved by the end of this project and conclusions and recommendations could be made.

This study found high concentrations of PAHs in wine, far above the recommended legal limit of Czech Republic. Volatile phenols were present in high concentrations in leaves, but showed no clear trend of distribution. Furthermore, only a few compounds were significantly for the taint associated with the creosote volatile emissions. A sensory panel could distinguish amongst wines, and associated the red wines in closest proximity to the stockyard with a burnt rubbery/tar taint. The volatile phenol results of the wines did not show the same trends as the panel described, and thus other compounds contributing to the associated taints and which is part of the emissions of creosote should be included for further studies to determine the compounds responsible for the creosote taint. These include styrene, biphenyl, benzene and toluene.

Furthermore low concentrations of PAHs were observed in soil, lower than was reported in other creosote contaminated sites. PAHs found in water were also lower than reported in literature (Wu, *et al.*, 2008). Leaves differed in concentrations and this was due to differences in species, location, lipid content and surface area of the leaves. Concentrations found in leaves were high for the vineyard and other surrounding species. Further research is needed to formulate a data base of creosote related compounds in the environment and in foodstuffs such as wine, grapes, apples, plums or any fruit grown with a creosote trellising system or on a polluted site. Various bio-remediation techniques and alternatives, as found in the literature, were discussed in detail in Chapter 2. An important insight from this research is that the effects of creosote emissions on wines made from contaminated grapes are obvious when analysed by a trained sensory panel and targeted chemical analysis.

The results obtained from the sensory and chemical analysis can be summarized as follows:

- The white and rosé wines were perceived as clean samples, whilst the red wines showed association with the burnt rubber/tar attribute and this association weakened as the distance from the stockyard increased. There were no difference between the

red wine treatments sensorially, except between the rosé (treatment A) and red wine styles (treatment B and C).

- The GC-MS results obtained showed the presence of most of the compounds analysed for. Vine leaves had the highest concentrations, followed by berries and wine, respectively, thus not all the compounds survive the fermentation process. Leaves differ according to the lipid content of the plant and the surface area- as was found by other authors. Wines had concentrations of m-cresol above the odour threshold in wine. For all the compounds measure the general trend was a decrease in from block 1 to 4. The red wine treatments were higher in concentration of compounds of interest that the red wines, this was the same for the sensorial analysis.
- The HPLC-DAD data obtained indicated that the concentration of chrysene and phenanthrene were high and ranged form 5 to 10 ppb, above the legal limit of 0.5ppb as determined by the Czech Republic. The soil and water data analysed showed low concentrations present in the surrounding environment. PAHs contamination varied according to soil depth and location (water and soil samples).

Sustainability is not only important for the industry, but is a growing consumer concern, therefore the environmental practices taken to ensure lower contamination levels in the environment as well as agricultural produce is of utmost importance. Creosote legislation should be updated in South Africa, along with PAH legal limits of foodstuffs and drinking water to be able to compete on an international level.

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6. Appendix

Addendum A

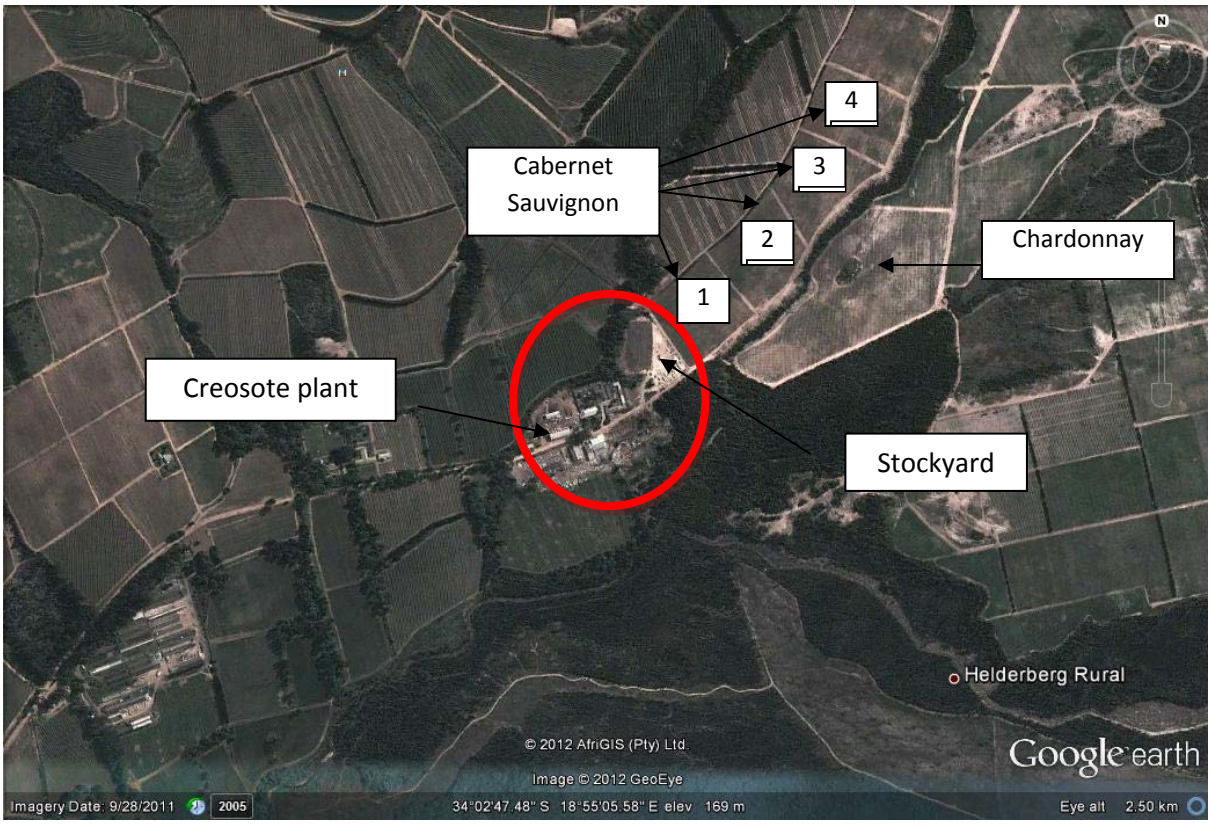

Figure 1: Vineyard layout of affected area