Polycyclic Aromatic Hydrocarbons: potential sources from the vineyard and analysis in wine

by

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SUMMARY

The South African wine industry plays an important role in the economy, contributing R36.1 billion gross domestic product (GDP) to the regional economy and employing 300 000 people both directly and indirectly in 2013. In order to build on this, the local industry continues to align itself to international trends and expectations. At the same time, polycyclic aromatic hydrocarbons (PAHs) are under continued scrutiny for their impact on the environment and health, due to their mutagenic and carcinogenic properties.

There has been some concern that grapes grown in South African vineyards may be exposed to various sources of PAH contamination. These sources of contamination include the usage of creosote posts in vineyards, bush fires and vehicle emissions, as well as the use of charred barrels in the cellar. The aim of this study was to determine if the exposure to PAHs resulted in the presence of PAHs in wine, and at which levels they may be present in wine.

Chapter 3 compared the use of the Ultra high-pressure liquid chromatography coupled with Fluorescence detection (UPLC-FLD) and the High-pressure liquid chromatography coupled with Fluorescence detection (HPLC-FLD) system for the analysis of PAHs in wine. Wine samples were extracted using the liquid-liquid extraction method developed by Panzeri (2013). It was found that both methods were suitable for low level PAH analysis, with the UPLC-FLD detector being faster.

In chapter 4, a selection of South African and international wines were analysed to determine the levels of PAHs found in the wines. The wines analysed had been randomly selected, from different regions, cultivars and vintages. Some wines had been made from grapes grown in vineyards using creosote posts for trellising and some were bush vines with no posts used at all. Some of the wines had been fermented or aged in oak barrels, while some had no wood treatment. The result of the analysis was that the majority of the wines selected had very low levels of PAHs. The levels were not high when compared to the legal limits set by the European Union of 1 ppm B[a]P for all dietary food (European Directive 2006/125/EC). The quantities of PAHs found in the wine samples were all below 2 ppb, which is the guideline set by AFFSA, the French Agency for Food, Environmental and Occupational Health & Safety (AFFSA, 2006). There were no differences between the PAH levels in South African and international wines.

Some wines analysed had levels of PAHs higher than the legal limit allowed in water of 0.01 µg/L for B[*a*]P and 0.1 µg/L for the sum of B[*b*]F, B[*k*]F, B[*ghi*]P and Ind[*1, 2, 3-cd*]P (European Directive

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2006/125/EC). This was potentially due to an accumulation of contamination from various sources – creosote, vehicle emissions, exposure to smoke from bush fires and extended barrel aging.

By eliminating creosote posts in the vineyard, avoiding bush fires just before harvest, as well as monitoring the use of charred oak barrels, the PAH levels should be reduced to levels below the legal limits in water. Further work could include looking at vineyards with high levels of PAHs in the soils and investigate how to reduce or remove the PAHs. The degradation of PAHs in wine over time could also be monitored.

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OPSOMMING

Die Suid-Afrikaanse wynbedryf speel ’n belangrike rol in die ekonomie en het in 2013 ’n bydrae van R36.1 biljoen bruto binnelandse produk (BBP) aan die streeksekonomie gemaak en tot 300 000 mense direk en indirek in diens gehad. Om hierop te kan bou, belyn die plaaslike bedryf homself voortdurend met internasionale tendense en verwagtinge. Terselfdertyd is polisikliese aromatiese koolwaterstowwe (PAK’e; *polycyclic aromatic hydrocarbons (PAHs)*) onder voortgesette noukeurige ondersoek vir hulle impak op die omgewing en op gesondheid as gevolg van hulle mutageniese en karsinogeniese eienskappe.

Daar is ’n mate van kommer dat druiwe wat in Suid-Afrikaanse wingerde groei, aan verskillende bronne van PAK besmetting blootgestel word. Hierdie bronne van besmetting sluit in die gebruik van teerpale in wingerde, veldbrande en uitlaatgasse van voertuie, sowel as die gebruik van vate in die kelder. Die doel van hierdie studie was om te bepaal of blootstelling aan PAK’e lei tot die teenwoordigheid van PAK’e in wyn, en teen watter vlakke hulle moontlik in die wyn teenwoordig is.

Hoofstuk 3 vergelyk die gebruik van ’n stelsel van ultra hoëdrukvloeistofchromatografie tesame met fluoressensie aftasting (UPLC-FLD) en hoëdrukvloeistofchromatografie tesame met fluoressensie aftasting (HPLC-FLD) vir die analise van PAK’e in wyn. Wynmonsters is geëkstraheer met gebruik van die vloeistof-vloeistof ekstraksiemetode wat deur Panzeri (2013) ontwikkel is. Daar is gevind dat beide metodes gepas was vir die analise van lae-vlak PAK, met die UPLC-FLD aanwyser wat vinniger was.

In hoofstuk 4 word ’n verskeidenheid Suid-Afrikaanse en internasionale wyne geanaliseer om die PAK-vlakke in die wyne te bepaal. Die wyne wat geanaliseer is, is lukraak gekies vanuit verskille streke, kultivars en oesjare. Sommige wyne is gemaak met druiwe vanuit wingerde wat teerpale gebruik as opleistelsel en ander was bosstokke waarvoor geen pale gebruik is nie. Sommige wyne is in eikehoutvate gegis of verouder, terwyl ander geen houtbehandeling ondergaan het nie. Die uitslag van die analises was dat die meerderheid wyne baie lae vlakke PAK’e bevat het. Die vlakke was nie hoog in vergelyking met die perke wat deur die Europese Unie bepaal is nie, naamlik 1 dpm B[a]P vir alle voedsel (European Directive 2006/125/EC). Die hoeveelhede PAK’e wat in die wynmonsters gevind is, was almal benede 2 dpb, wat die riglyn is van die AFFSA, die Franse Agentskap vir Kos-, Omgewings- en Beroepsgesondheid en Veiligheid (AFFSA, 2006). Daar was geen verskille in die PAK-vlakke tussen die Suid-Afrikaanse en internasionale wyne nie.

Sommige van die geanaliseerde wyne het PAK-vlakke gehad wat hoër was as die wettige perk wat in water toegelaat word, van 0.01 µg/L vir B[*a*]P en 0.1 µg/L vir die som van B[*b*]F, B[*k*]F, B[*ghi*]P en Ind[*1, 2, 3-cd*]P (European Directive 2006/125/EC). Dit is potensieel as gevolg van die ophoping van besmetting vanaf verskillende bronne: teer, uitlaatgasse van voertuie, blootstelling aan rook vanaf veldbrande en verlengde vatveroudering.

Deur teerpale uit wingerde te verwyder, veldbrande kort voor oes te vermy, sowel as die gebruik van eikehoutvate te monitor, sal PAK-vlakke moontlik verlaag word tot vlakke benede die wettige perk in water. Verdere werk sou die bestudering van wingerde met hoë vlakke van PAK’e in die grond

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kon insluit, tesame met ondersoeke oor hoe die PAK'e verminder of verwyder kan word. Die afbreking van PAK'e in wyn oor tyd kan ook gemonitor word.

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This thesis is dedicated to my family for their support and encouragement

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BIOGRAPHICAL SKETCH

Anne Alessandri was born on 6 January 1975 and matriculated from Paarl Vallei High School in Somerset West, South Africa in 1992. After studying Tourism Management and Development, Anne worked as a graphic designer for 7 years. She then obtained her BScAgric-degree at Stellenbosch University in 2007, majoring in Viticulture and Oenology. Anne completed her HonsBScAgric-degree in Viticulture in 2008. While working at Stellenbosch University as the Liaison officer for the internship program, Anne enrolled for an MScAgric-degree in Oenology.

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PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the South African Journal of Enology and Viticulture.

Chapter I **General introduction and project aims**

Chapter II **Literature review**
Potential sources of Polycyclic Aromatic Hydrocarbons (PAHs) in the vineyard and in wine

Chapter III **Research results**
Comparison of UPLC and HPLC methods for determination of Polycyclic Aromatic Hydrocarbons (PAHs) in wine

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Chapter 1

Introduction and project aims

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CHAPTER I: INTRODUCTION AND PROJECT AIMS

1.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds consisting of two or more fused aromatic rings. Approximately 250 different PAHs have been identified, and are differentiated into two groups based on their size. 'Light' PAHs consist of three or four aromatic rings and 'heavy' PAHs have at least five aromatic rings (Agency for Toxic Substances and Disease Registry, 1995). The heavy PAHs are known for their strong mutagenic, carcinogenic and toxic properties. Light PAHs, although not carcinogenic are known to pose toxic effect to many aquatic organisms (Brown and Peake, 2006). PAHs are not yet classified as persistent organic pollutants (POPs) under the Stockholm Convention; they are nonetheless ubiquitous environmental contaminants that are resistant to degradation and can remain in the environment for long periods with the potential to cause adverse environmental effects.

Creosote posts, commonly used in vineyards in the Western Cape, are pine posts treated with coal tar creosote to preserve the wood against climatic and biological degradation. The major chemicals in coal tar creosote are PAHs, phenol, and creosols. It has been proven that PAHs leach into soil and water, and have various negative environmental impacts, as well as negative impacts on the health of people who are directly or indirectly exposed to PAHs (Agency for Toxic Substances and Disease Registry, 1995).

Panzeri (2013) examined the influence of vineyard post type on the chemical and sensorial composition of Sauvignon blanc and Merlot noir wines made from grapes grown near the posts. The overall aim of this study was to determine if there is a relationship between creosoted posts usage and 'burnt rubber' taint in wine. Panzeri found a direct link between wines produced by grapes grown next to the new creosote poles, and 'burnt rubber', 'phenolic', 'smoky' and 'medicinal' characteristics. Although Panzeri's study did not find significant amounts of PAHs in wines made from creosote-exposed grapes, a screening of commercial South African wines by HPLC-DAD done at the same time indicated that there were PAHs present in wine at levels that exceeded the recommended legal limits in food, set by the Commission Regulation (EU) No 835/2011. The PAH contamination in wine may have been as a result of the vines exposure to creosote posts, but the cause of the contamination was not confirmed. The aim of this study is to review potential sources of PAHs found in the vineyard and in wine, and to compare the HPLC-FLD and UPLC-FLD as analytical tools for determination of PAHs in wine. The study will look at the levels of PAHs found in wine to determine if the levels exceed the recommended levels of PAHs in food using validated chromatographic methods.

1.2 Project Aims

The aim of the literature review is to investigate the various sources of PAHs that may contaminate wine. A further aim is to determine if PAHs are present in both South African wine and international wines, and in what quantities. There are many PAH compounds in the environment,

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but for the purpose of this study, the focus is limited to 12 of the 16 priority PAHs, as classified by the European Commission Regulation, 2005, that were readily available for the study. These 12 PAHs include the eight PAHs used as indicators of the carcinogenic potency of PAHs in food (EFSA, 2007). Wines will be tested for these 12 priority PAHs, to determine which are present in wine, and whether they are above the legal recommended levels in food (set by the European Commission). The European Commission has no legal limits set for wine.

Aim 1: Determine the potential sources of PAHs found in wines

Aim 2: Determine suitability of HPLC-FLD and UPLC-FLD as analytical tools for analysis of PAHs in wine

> **Objective:** Compare HPLC-FLD and UPLC-FLD as analytical tools for the quantification of PAHs in wine

Aim 3: Assess levels of PAHs in South African and international wines

1.3 Significance of the research

Limited research has been conducted on PAHs in the wine industry. The results have been variable and some have been cause for concern. The purpose of this study is to give an overview of PAHs related to the wine industry, and investigations will include South African and international wine samples. With a broader understanding of potential sources of PAHs in the industry, one might get closer to learning how best to reduce PAHs in the agricultural environment. A random selection of wines will be analysed for PAHs to determine if the levels of PAHs found in wine are significant. Known sources of PAHs within the environment will be discussed to determine potential sources of PAH contamination in wine.

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Chapter 2

Literature review

Potential sources of Polycyclic Aromatic Hydrocarbons in the vineyard and in wine

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CHAPTER II: POTENTIAL SOURCES OF PAHS IN THE VINEYARD AND IN WINE

2.1 Introduction

The wine industry is very important to the South African economy, contributing R36.1 billion gross domestic product (GDP) to the regional economy and employing 300 000 people both directly and indirectly in 2013. South Africa ranks as number seven in the world in overall volume production of wine and produces 4.2% of the world's wine (2014) (SAWIS, 2015).

Exports of natural (*i.e.* non-fortified) packaged wines for the 2014 calendar year reached 173.4 million litres. Total exports of wine in 2014 were 422.7 million litres.

The local wine industry continues to align itself to international trends, expectations and legislation. At the same time, polycyclic aromatic hydrocarbons (PAHs) are under continuous scrutiny as their impact on the environment and human health is of growing concern due to their mutagenic and carcinogenic properties (Harvey, 1991).

There has been some concern that grapes grown in South African vineyards may be exposed to various sources of PAHs. These sources include the use of creosote posts in vineyards, bush fires and vehicle emissions, all of which are known to cause environmental contamination by PAHs. This review will also investigate various sources of PAH contamination during the wine making process (for example, the use of barrels, oak chips, cork or contamination by vehicle emissions. The aim of this study is to determine if this exposure of grapes and wine to PAHs has resulted in the presence of PAHs in wine, and at which levels they may be present in wine.

2.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are a group of organic compounds consisting of two or more fused aromatic rings. They are organic contaminants that are resistant to degradation, can remain in the environment for long periods and have the potential to cause adverse environmental effects (EFSA, 2008). PAHs are contaminants which may occur in all parts of the environment: atmosphere, inland and sea waters, sediments, soils and vegetation.

PAHs are formed both naturally and due to anthropogenic sources. Nowadays, PAHs are formed mainly as a result of anthropogenic processes, particularly from incomplete combustion of organic fuels. Anthropogenic sources of PAH include burning of fossil fuel, coal tar, wood, garbage, refuse, used lubricating oil and oil filters, municipal solid waste incineration and petroleum spills and discharge. Natural processes, such as volcanic eruptions and forest fires, also contribute to an ambient existence of PAHs. PAHs are widely distributed in the atmosphere.

PAHs can be present in both particulate and gaseous phases, depending upon their volatility. Low molecular weight PAHs (LMW PAHs) have two or three aromatic rings and are predominantly emitted in the gaseous phase. PAHs with four rings exist both in the vapour and particulate phase while high molecular weight PAHs (HMW PAHs), with five or more rings, are emitted in the particulate phase (Yebra-Pimentel *et al.,* 2013). In the atmosphere, PAHs can undergo photo-degradation and react with other pollutants, such as sulphur dioxide, nitrogen oxides, and ozone. Due

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to widespread sources and persistent characteristics, PAHs disperse through atmospheric transport and exist almost everywhere. For this reason, human exposure to PAHs in gaseous or particulate phases is virtually unavoidable. This is a cause for concern as long-term exposure to high concentrations of PAHs is associated with adverse health problems (EFSA, 2008). PAHs are known for their strong mutagenic and carcinogenic properties and are potent immunosuppressants. PAHs are lipophilic and bioaccumulative. The most significant endpoint of PAH toxicity is cancer (ATSDR 1995, 2009).

2.3 Priority Polycyclic Aromatic Hydrocarbons

Over 250 PAH compounds have been identified. The United States Agency for Toxic substances and Disease Registry has listed 17 priority PAHs, based on their occurrence and carcinogenicity. Although their toxicity varies, they are all considered to be more harmful that other PAHs. The Environmental Protection Agency (EPA) lists 16 priority PAHs (Environmental Protection Agency, 1983) as seen in Table 1. Table 1 also lists which of these PAHs are monitored by the EU Scientific Committee for Food (SCF), the European Union (EU), and the US Environmental Protection Agency (EPA) based on their toxicity in food.

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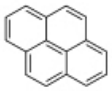
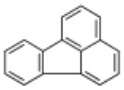
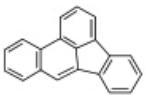
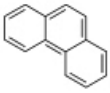
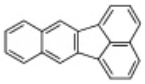
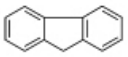
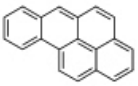
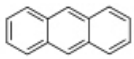
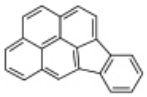
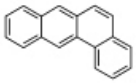
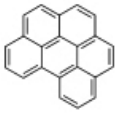
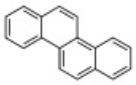
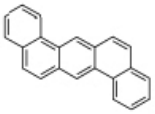
Table 2.1: *Structures and nomenclatures of the 16 PAHs on the US Environmental Protection Agency priority pollutant list, frequently monitored according to recommendations by the EU Scientific Committee for Food (SCF), the European Union (EU), and the US Environmental Protection Agency (EPA). (Adapted from Wang et al., 2006 and Lerda, 2011)*

List	Common Name	Structure	List	Common Name	Structure
EPA	Naphthalene		EPA	Pyrene	
EPA	Acenaphthene		EPA	Fluoranthene	
EPA	Acenaphthylene		EPA, SCF, EU	Benzo[*b*] fluoranthene	
EPA	Phenanthrene		EPA, SCF, EU	Benzo[*k*] fluoranthene	
EPA	Fluorene		EPA, SCF, EU	Benzo[*a*] pyrene	
EPA	Anthracene		EPA, SCF, EU	Indeno[1,2,3-*cd*]pyrene	
EPA, SCF, EU	Benz[*a*] anthracene		EPA, SCF, EU	Benzo[*ghi*] perylene	
EPA, SCF, EU	Chrysene		EPA, SCF, EU	Dibenz[*a,h*] anthracene	

2.4 Physical and Chemical Characteristics of PAHs

Polycyclic aromatic hydrocarbons refer to a large class of organic compounds containing two or more fused aromatic rings made up of carbon and hydrogen atoms. They are white/pale yellow solids with high melting- and boiling-points, low vapour pressure, and very low water solubility which decreases with increasing molecular mass.

PAH are soluble in many organic solvents (IARC, 1983) and are highly lipophilic. In aqueous environments, PAHs are mostly found adsorbed on particulates or on humic matter. They can also be found dissolved in oily contaminant that may be present in the water, sediment or soil. PAHs are chemically inert. Reactions of importance in terms of agriculture are photolysis and reactions with nitrogen oxides, nitric acid, sulphur oxides, sulphuric acid, ozone, and hydroxyl radicals, as this leads to their degradation.

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2.5 General Environmental Sources of PAHs

The emission of PAHs from various sources can be divided into the following five categories according to the PAH Positions Paper 2001 (OOPEC, 2001): domestic, mobile, industrial, agricultural and natural sources.

2.5.1 Domestic emissions

Domestic emissions include heating and cooking. The burning of various fuels for heat (coal, oil, gas, wood or garbage) as well as various cooking methods (grilling or smoking food) contribute to the quantity of PAHs present in the atmosphere. Domestic emissions will vary depending on different climates and the heating system used. Warmer areas rely less on heating than colder regions. For the same reason, levels of PAH in the atmosphere are higher in the winter than in the summer period (Yang Y *et al.*, 2010). Cigarette smoke is also an important source of PAHs in indoor environments.

2.5.2 Mobile emissions

Mobile emissions include emissions from various vehicles: cars, ships, aircrafts, trains, *etc*. This category could also include other machinery such as generators and pumps. PAH emissions from mobile sources are associated with use of diesel, coal, gasoline, oils, and lubricant oil. Emission is a function of engine type, emission control, load, age, fuel and driving mode, including cold starting (Ravindra *et al*., 2007). According to Ravindra, the largest contributor to PAHs in urban area is exhaust emissions from petrol and diesel vehicles. A further contributor to PAH emission are tyres – the abrasion of tyres on tar roads results in the release of PAHs. All mobile sources contaminate air, soil and water present near the source of emission.

2.5.3 Industrial emissions

Industrial sources of PAHs come from primary aluminium production, coke (fuel) production, creosote and wood preservation, waste incineration, cement manufacture, petrochemical and related industries, bitumen and asphalt industries, rubber tyre manufacturing and commercial heat / power production. Industrial sources are being increasingly regulated, resulting in a decrease in total PAH emissions from this source. Further pollution can be caused by oil spillages or leaching of coal tar products (Bedient, 2004). PAHs enter surface waters through storm water runoff, discharges from industries and wastewater treatment plants.

Although the pyrolysis of scrap tyres is not an industrial source, it is an important source to consider in South Africa. South Africa generates 160,000 tons of scrap tyres per year, and although there is legislation in place prohibiting burning of tyres in an open space, the problem continues unabated due to insufficient enforcement (Mahlangu, 2013).

2.5.4 Agricultural emissions

Agricultural sources include smoke from any fires that involve burning organic materials under sub-optimum combustion conditions (Radojevic, 2003; Kennison, 2009). A further source of

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contamination of both soil and water is from the spreading of sewage sludge on agricultural fields (Hembrock-Heger and Konig, 1990 as cited by IPCS, 1998).

2.5.5 Natural sources

Natural sources of PAHs include smoke from natural fires (due to lightning strikes, *etc*) and volcanic eruptions. PAHs can also be formed in nature in three ways: the high temperature pyrolysis of organic materials; low to moderate temperature diagenesis of sedimentary organic material to form fossil fuels; direct biosynthesis by microbes and plants (Neff, 1979). There is very little literature estimating the concentration of PAHs in the environment resulting from natural sources of PAHs emission.

It is clear from the domestic, mobile, industrial and agricultural emission sources listed, that PAH compounds will readily be found in contaminated water, soils and air, as well as certain food products such as fish, smoked products, fruits and vegetables grown in vicinity of contaminated sites.

2.6 International and National legislation

International legislation of PAHs found in food is regulated by the EU Scientific Committee for Food (SCF), the European Union (EU), and the US Environmental Protection Agency (EPA) (Lerda, 2011). The EU Commission has set various maximum levels for PAHs in certain foodstuffs, with a focus on foods that contain fats and oils or where the food is smoked (Table 2). Originally, benzo[*a*]pyrene was chosen as a marker for the occurrence and effects of carcinogenic PAHs in food. The US EFSA Panel on Contaminants in the Food Chain reconsidered this in 2007, and in 2008, it was decided that the sum of 8 PAHs (benzo[*a*]pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene, chrysene, dibenz[*a,h*]anthracene and indeno[*1,2,3-cd*]pyrene) or '8PAH' was a better indicator of the carcinogenic potency of PAHs in food. Further studies revealed that the sum of benzo[*a*]pyrene, chrysene, benz[*a*]anthracene and benzo[*b*]fluoranthene ('4PAH') is as good an indicator to use as 8PAH since the correlation between 4PAH and 8PAH was 0.99 (EFSA, 2007). Table 3 gives a list of the 8PAH and their abbreviations.

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Table 2.2: *Maximum acceptable levels of B[a]P and 4PAH in foodstuffs (adapted from The Commission of the European Communities, 2006).*

Foodstuffs		**Maximum levels (µg/kg)**	
		Benzo(*a*)pyrene	Sum of benzo(*a*)pyrene, benz(*a*)anthracene, benzo(*b*)fluoranthene and chrysene
1	Oils and fats (excluding cocoa butter and coconut oil) intended for direct human consumption or use as an ingredient in food	2.0	10.0
2	Cocoa beans and derived products	5.0 µg/kg fat	30.0 µg/kg fat
3	Coconut oil intended for direct human consumption or use as an ingredient in food	2.0	20.0
4	Smoked meat and smoked meat products	2.0	12.0
5	Muscle meat of smoked fish and smoked fishery products, excluding fishery products listed in points 6.1.6 and 6.1.7. The maximum level for smoked crustaceans applies to muscle meat from appendages and abdomen. In case of smoked crabs and crab-like crustaceans (Brachyura and Anomura) it applies to muscle meat from appendages.	2.0	12.0
6	Smoked sprats and canned smoked sprats; bivalve molluscs (fresh, chilled or frozen) ; heat treated meat and heat treated meat products sold to the final consumer	5.0	30.0
7	Bivalve molluscs (smoked)	6.0	35.0
8	Processed cereal-based foods and baby foods for infants and young children	1.0	1.0
9	Infant formulae and follow-on formulae, including infant milk and follow-on milk	1.0	1.0
10	Dietary foods for special medical purposes intended specifically for infants	1.0	1.0

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***Table 2.3**: The list of PAHs making up '8PAH', with their abbreviations.*

PAH	**Abbreviation**
benzo[*a*]pyrene*	B[*a*]P
Chrysene*	Chy
benz[*a*]anthracene*	B[*a*]A
benzo[*b*]fluoranthene*	B[*b*]F
benzo[*k*]fluoranthene	B[*k*]F
dibenz[*a,h*]anthracene	DB[*a,h*]A
indeno[*1,2,3-cd*]pyrene	Ind[*1, 2, 3-cd*]P
benzo[*ghi*]perylene	B[*ghi*]P

*PAHs that are part of the '4PAH' group

In South Africa, the legislation for PAHs in food follows the European Union guidelines. However, in terms of agriculture, a potential source of PAH contamination in vineyards investigated by Van Zyl (2013), is the use of creosote posts for trellising of grapevines. Van Zyl found that the stockyard had negative effects on the surrounding environment in terms of sensory and chemical contamination. Although the treatment and use of wooded posts has to comply with SABS 457-3:2013 (ISBN 978-0-626-29461-8) Edition 7.1, which encompasses the preservative treatments and includes the use of creosote, there is no restriction for the use of coal tar creosote as wood preservative. Creosote is readily available and easily purchased (Van Zyl, 2013). The Integrated Production of Wine (IPW) certification system suggests the use of creosote alternatives for wood preservation for use in vineyards (http://www.ipw.co.za). This is however not legislated as the IPW is a voluntary producer organisation.

2.7 PAHs in foodstuffs

PAHs are not normally found in raw food (European Commission, 2002). They are usually formed by processing, smoking, roasting, baking or frying food. Raw vegetables can however be contaminated by the deposition of airborne particles or by growth in contaminated soil (European Commission, 2002). The permitted levels of individual PAHs in meat, fish, dairy products, vegetables and fruits, cereals and their products, sweets, beverages, and animal and vegetable fats and oils are in the range of 0.01-10 µg/kg (or parts per billion) (Table 2). However, concentrations of over 100 µg/kg have been detected in smoked meat and up to 86 µg/kg in smoked fish; smoked cereals contained up to 160 µg/kg. Coconut oil has been found to contain up to 460 µg/kg. The levels in human breast milk can range from 0.003-0.03 µg/kg (IPCS, 1998).

The intake of individual PAHs from food has been estimated to be 0.10-10 µg/day per person. The total daily intake of B[*a*]P from drinking-water was estimated to be 0.0002 µg/person (EFSA, 2008). Cereals and cereal products are the main contributors to the intake of PAH from food because they are a major component of the total diet (EFSA, 2008).

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The limits for PAHs in drinking water intended for human consumption has been set by EU Council Directive 98/83/EC at 0.01 ppb for B[*a*]P and 0.1 ppb for the sum of B[*b*]F, B[*k*]F, B[*ghi*]P and Ind[*1, 2, 3-cd*]P. Alcoholic beverages are not included in this legislation. The only country to have a legal limit for PAHs in wine is the Czech Republic, with a maximum limit of 0.5 ppb PAHs (the sum of B[*a*]A, B[*b*]F, B[*k*]F, DB[*a,h*]A, dibenzo(*a,h*)pyrene, dibenzo(*a,i*)pyrene, Ind[*1, 2, 3-cd*]P and chrysene) (Wenzl, *et al.*, 2006). The European Union has a set legal limit of 1 ppm B[*a*]P for all dietary food (European Directive 2006/125/EC). AFFSA, the French Agency for Food, Environmental and Occupational Health & Safety has guidelines of 2.0 ppb of wet weight for wine (AFFSA, 2006).

2.8 Potential sources of PAHs in agriculture

In general, agricultural land may be exposed to PAHs through two main sources – contaminated water used for irrigation and atmospheric pollution. The concentrations of PAHs in soil produced by natural processes (*e.g.*, vegetation fires, volcanic activity) are estimated to be around 1-10 ppb (Wilcke, 2000). However, Wilcke also found that various agricultural soils that have been exposed to PAH contamination have concentrations of up to 10 times higher than this.

A study in Tianjin, China, by Tao *et al.* (2004) examined the effect that contaminated soils and water has on vegetables grown in these soils. The site in question was located immediately next to an urban district that has been irrigated with wastewater for several decades. They measured various priority PAHs in the soil and in vegetables. The 8PAH levels in the contaminated soils ranged from 330 to 2200 ppb. The average 8PAH levels in aerial parts of the vegetables ranged from 29 to 70 ppb and exceed the limitation values recommended by the European Union of 1 ppb for vegetables (The Commission of the European Communities, 2006).

A further study done by Pucareviã and Sekuliã (2004) analysed soil samples from non-agricultural soils, agricultural soils, industrial soils and urban soils. They found that PAH levels were highest in industrial soils, followed by urban soils and then agricultural soils. By comparison, the soils from non-agricultural soils (nature reserves) had very low levels of PAHs. Higher levels of PAHs were found in soils closer to roads and industrial activity. Studies are currently being conducted at Stellenbosch University to determine the uptake of PAHs from contaminated soils into the grapevine and the subsequent contamination of wine.

Atmospheric pollution can be as a result of industry emissions and the proximity of the agricultural land to industry sites. It is however more often the result of burning of biomass. Burning of agricultural waste to prepare land or for regenerative purposes is a fairly common practice. The organic material is usually burned under sub-optimal conditions, resulting in a significant production of PAHs (Ravindra *et al.*, 2008).

2.8.1 Vineyard sources of PAH contamination

In terms of the wine industry, there are various ways in which grapes can potentially be contaminated by PAHs. This could occur either in the vineyard or in the cellar. One of the sources is the coal-tar creosote used as a preservative for wooden agricultural posts. In a study conducted by Van Zyl (2013),

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it was shown that creosote-treated posts had a negative impact on the environment (soil, water and grapes found close to creosote post stockpiles). Leaching of creosote from the stockpiles into the soil and groundwater has caused increased levels of PAHs present compared to areas further away from the stockpiles. Creosote treated vineyard posts are commonly used in the Western Cape in the wine industry, and have been for decades. Creosote has been used to protect wood from attack by fungi, marine borers, and insects in the United States since 1865. It is a distillate derived from coal tar produced by the carbonisation of bituminous coal. Creosote is a complex mixture of at least 160 detectable hydrocarbon compounds, and all 18 major components are cyclic and aromatic (Brooks, 2004). The American Wood Preservation Association describes creosote as:

> *"a distillate of coal-tar produced by high temperature carbonisation of bituminous coal; it consists principally of liquid and solid aromatic hydrocarbons and contains appreciable quantities of tar acids and tar bases; it is heavier than water, and has a continuous boiling range of approximately 275°C, beginning at about 175°C. Polycyclic aromatic hydrocarbons make up approximately 90% of the mass of coal tar" (AWPA, 1977).*

Grapes grown in vineyards using creosote posts may be at risk of possible PAH contamination. This could be through contamination of the fruit by volatile PAHs adhering to the waxy cuticle of the grape skins, or it could potentially be contamination by means of uptake by above-ground parts of the plant or uptake through the roots. The exact mechanism for how PAHs are taken up by roots in vines is still being researched. It has however been proven that other plants such as wheat (Zhan *et al*., 2010), cauliflower, celery, white turnip (Tao *et al*, 2003) and lettuce (Khan *et al*, 2008) are able to incorporate PAHs through up take by the roots.

Further contamination could be as a result of the proximity of the vineyard to sources of mobile emissions. This can result in soil contamination as well as direct contamination of the grapes by PAHs in their particle form. The closer a vineyard is to a railway, busy motorway, urban area or industrial activity, the larger the risk of contamination through vehicle exhaust and industrial emission.

The most common source of PAHs in the environment is the incomplete combustion of biomass (such as wood) and fossil fuels (petroleum and coal) (Lima, *et al.*, 2005). Bushfires in close proximity to vineyards is a relatively wide spread phenomenon in wine industries worldwide. Australia lost 54.5 million hectares of forest and grass to fire in 2000, and with climate changing, this is expected to increase annually (Krstic *et al.*, 2015). For this reason, extensive research has been done in Australia on bush fires and the impact fire has on the quality of the wine. The focus of the research has to date been on the negative effect smoke has on the wine aroma. Affected wines are characterised by undesirable sensory characters and are described by Krstic *et al.* (2015) as smoky, burnt, ash, smoky bacon, medicinal and ash tray. Volatile phenols have been identified as the compounds responsible for smoke taint in wine. No studies have looked specifically at the impact fire or smoke may have on the PAH levels in wine.

The Western Cape is notorious for bush fires during the summer months. These fires are often in agricultural areas, and vineyards are directly affected by the smoke and soot deposition.

Ultimately, PAHs from creosote, air pollution, from fires and from polluted water are deposited onto the soil and onto the grapes.

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2.9 Source identification of PAHs

During the different processes that release PAHs into the environment, the concentration and ratio of certain PAHs can be used to determine the contribution of the difference sources to their concentration in air (Ravindra *et al.*, 2008). These PAHs are called source markers, tracers or signatures. A study by Li and Kamens (1993) categorised the PAH signatures for 3 combustion sources (residential wood combustion, gasoline spark ignition emissions and diesel engine emissions).

Khalili *et al.*, (1995) determined the signatures for the major sources of airborne PAHs in Chicago. Table 4 summarises the findings. They found that 2- and 3-ring PAHs are responsible for a large percentage of PAHs found in tunnels, from diesel and petrol engines, coke ovens and wood combustion.

Table 2.4: *Source distribution of percentage PAHs to total mass (Khalili et al., 1995).*

(Bdl: below detection limit)

PAH	Highway tunnel	Diesel engines	Petrol engines	Coke oven (coal)	Wood Combustion
2-Ring	76.2	8.7	55.6	89.8	11
3-Ring	16	56.2	18.1	8.9	69.2
4-Ring	4.3	10.8	12.6	0.97	6.6
5-Ring	3.1	18.7	13.4	0.22	13.2
6-Ring	0.4	5.2	0.05	0.01	Bdl
7-Ring	Bdl	0.2	0.08	Bdl	Bdl

Various studies have subsequently been done to determine if individual or groups of PAHs can be used as source markers. There is sufficient evidence to suggest that this is possible, and by using Principal Component Analysis (PCA) to analyse data, one can determine the likely source of emission by knowing the markers present. It would be useful if the source of PAH contamination in wine could be identified by means of the type of PAHs found.

2.10 Potential sources of PAHs in the cellar

2.10.1 Oak barrels and chips

There are number of potential sources of PAH contamination of wine in the cellar, ranging from winemaking practices to exposure to barrels and oak chips during maturation and aging, environmental contamination or the use of cork closures.

Oak barrels are regularly used for the maturation of wine. During the process of manufacturing these barrels, the oak is toasted, resulting in an increase of temperature and pyrolysis of the oak wood. This causes modifications in the physical structure and chemical composition of the wood, with the resultant formation of PAHs as part of a large range of pyrolysis products of lignin (Garcia-Falcon and Simal-Gandara 2005). These PAHs remain in the pyrolised wood until they are later extracted by wine in direct contact with the barrel.

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Garcia-Falcon and Simal-Gandara (2005) investigated the levels of PAHs found in alcoholic beverages that were exposed to wine barrels. The study included the analysis of off-the-shelf alcoholic drinks, different wood used for barrels (American versus French oak), the types of charring (traditional and convective) used and the charring intensity (light, medium and heavy toasting). The wine analysed had low levels of PAHs present, all below that of the maximum amount allowed in drinking water of 0.1 µg/L for the sum of the four PAHs (B[*b*]F, B[*ghi*]P, B[*k*]F and I[1,2,3-*cd*]P) (European Commission 1998).

The traditional heating processes associated with barrel production resulted in the formation of various PAHs in the wood. This occurred on the surface of the barrel (directly exposed to combustion), as well deeper into the staves, as these PAHs migrate into the wood. However, no carcinogenic PAHs were present in significant quantities. As the toasting intensity increased, the levels of PAHs increased – although they still remain below the levels allowed in drinking water and the B[*a*]P levels were below the guideline value set by AFFSA of 2µg/kg fresh weight (*i.e.*, 2000 ng/L of wine). Wood charred by convection method found no PAHs present.

Chatonnet and Escobessa (2007) found similar results to Garcia-Falcon and Simal-Gandara (2005). They studied the effect of PAH extraction of the same barrel over three years. The extraction of PAHs from barrels into wine remained the same in the first and second year of use. Only after the third year did the levels of PAHs extracted into wine decrease. This was attributed to the low solubility of PAHs and because the PAHs are located not only on the surface of the barrel, but in the deeper layers as well.

The PAH content of wine ages in American oak barrels was found higher than that of French Oak barrels, due to the longer toasted time required for this type of wood (Chatonnet and Escobessa, 2007).

It was concluded in both studies that although the level of PAHs was highest in wine exposed to heavily toasted barrels made using the traditional charring method, the levels of carcinogenic PAHs remained below the legal limits allowed in water. Garcia-Falcon and Simal-Gandara (2005) recommend that any health hazards can be minimised by using convective toasting to manufacture barrels where the drinks are to be aged.

Oak chips are increasingly being used by winemakers to replace oak barrels for economic reasons. Chinnici *et al.* (2006) determined the presence of PAHs in wood chips used in the production of wine. Samples of 15 commercially available batches of wood chips used in the alcoholic beverage industry were extracted and analysed for their PAH content. The samples included wood chips from French and American oak, wood chips of different sizes and of different toasting levels. Unlike wine barrels, wood chips are processed using air-dried oak and not traditional charring.

PAHs were found in all the wood chip samples in very low amounts, below that of the maximum amount allowed in drinking water (European Commission 1998). The origin, toasting level and size of the chips made little difference to the PAHs found.

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2.10.2 Cork stoppers

There is a risk that cork can become environmentally polluted during the long periods spent in open air (during growth and bark storage). Wine stoppers made from the cork can be in direct contact with wine for periods ranging from a few months to many years. It is therefore important that the stoppers are free from PAHs. In a study by Mazzoleni *et al*, (2005) corks were tested for various persistent organic pollutants, including for PAHs. It was found that cork is able to absorb selective volatiles such as naphthalene from the environment. These volatiles are able to partially migrate into wine when the cork is used to seal a bottle of wine. The study only analysed for phenanthrene, anthracene and fluoranthene (LMW PAHs) and these were found to be at levels consistent with values found in food and therefore safe to use. These three PAHs are not part of the 8PAH or 4PAHs used as indictors for carcinogenic potency of PAHs in food (EFSA, 2008).

2.10.3 Winemaking practices

Grapes can be exposed to environmental contamination of PAHs by exposure to creosote posts, mobile emissions or bush fires. The contamination of wine from the grapes could be caused by certain wine making practices such as cold soaking or extended skin contact. Panzeri (2013) examined the impact of creosote on the PAH levels of wine made from grapes grown immediately next to new creosote posts. Wines were made from Sauvignon blanc and Merlot noir grapes. Despite the exposure to creosote, there was no significant increase in PAH levels for these wines. This could be as a result of the hydrophobicity of PAHs, resulting in a poor transfer rate of these compounds from the skins to the juice (Rey-Salguera *et al*, 2004).

2.10.4 Other sources

Machinery in cellars that use petrol, diesel or oil may contaminate the grapes or wine with PAHs, either through their exhaust emissions or possibly directly by oil leaking directly onto grapes or into wine. There is no literature available investigating this. However, the study by Chatonnet and Escobessa (2007) investigated the PAH levels in wood used for production of barrels. Usually high levels of PAHs were found in air-dried stave wood stored in a final conditioning unit. PAH levels were expected to be low, with no carcinogenic PAHs present. Further investigation proved that the accumulation of the PAHs on the surface of the microporous stave wood was due to the exhaust gases of the diesel-engine forklift trucks used to move the wood into the conditioning units, where the doors were kept closed during loading.

2.11 Conclusions

PAHs can potentially end up in wine through a number of sources. Soil contamination (caused by the use of polluted water, vehicle or industry emissions or the leaching of PAHs from creosote posts) can potentially result in the uptake of PAHs though the roots, into the vine and subsequently into the grape itself. Studies are currently being conducted to investigate whether PAHs are able to be taken up by the grapevine. A further source of contamination is through the physical deposition of PAHs

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onto the grape skin (volatile PAHs from creosote posts or from soot and smoke from bush fires) or from cellar practices (the use of heavily toasted wooded barrels or oak chips, maceration on contaminated skins), environmental contamination from exhaust emissions, various winemaking practices, *etc*).

In order to ensure that PAHs are kept to a minimum in wine, it is important to identify potential sources and eliminate or minimise the impact that they have on wine. Affected soils need to be remediated in order to facilitate the breakdown or removal of PAHs. The use of creosote posts needs to be carefully monitored within agriculture in general. Should the use of creosote in South African vineyards be seen as a potential health threat by the international market, it is important to demonstrate that the PAH levels in South African wines fall well below that of the suggested legal limits. It is also important that South Africa aligns itself with the accepted international practices, thus moving away from creosote treated posts towards alternative treatments.

Air and water pollution from industrial sites is more difficult to control – however, there is pressure on big industries to reduce their air pollution, thus reducing this source.

Cellar practices can be monitored to ensure all is done to avoid any environmental contamination. The use of heavily toasted barrels can be evaluated to assess contribution to the PAH levels in wine. The impact seasonal bush fires have on the grapes can also be assessed as necessary, to ensure the contribution to the overall PAH levels is within the recommended legal limits.

The guidelines from AFFSA give the maximum limits of B[*a*]P allowed in alcoholic beverages of 2.0 µg/kg. If wines are found to have levels of PAHs above this level, they may be highlighted as a health concern and taken off the shelves.

Studies have been done worldwide to identify the sources of PAHs and monitor the levels of contamination in the environment. Legislation has been implemented internationally to ensure that PAHs in foodstuffs and drinking water does not exceed dangerous limits, and there have been significant attempts to reduce the emission of PAHs where possible.

South Africa does not have legislation in place to regulate sources of PAH emission. It is important in terms of the wine industry to recognise the various sources of PAHs in order to eliminate or reduce them.

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Chapter 3

Research results

Comparison of UPLC and HPLC methods for determination of Polycyclic Aromatic Hydrocarbons (PAHs) in wine

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CHAPTER III: COMPARISON OF UPLC AND HPLC METHODS FOR DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) IN WINE

Abstract

Ultra high-pressure liquid chromatography (UPLC) and high-pressure liquid chromatography (HPLC) methods for determination Polycyclic Aromatic Hydrocarbons (PAHs) were compared for speed and the proficiency in detecting low levels of PAHs in wine. Sample preparation was carried out using a validated liquid-liquid extraction method (LLE) Panzeri, (2013). Both instrumental methods are relatively rapid: total running time was 29,48 minutes and 17,90 minutes for HPLC and UPLC methods, respectively. In this study, methods were validated in terms of limits of detection (LOD) and quantification (LOQ), accuracy and recovery. Both methods were found to be sensitive and suited for the analysis of PAHs in wine.

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds consisting of two or more fused aromatic rings. They are organic contaminants that are resistant to degradation, can remain in the environment for long periods and have the potential to cause adverse effects in biological organisms. PAHs occur in all parts of the environment: atmosphere, inland and sea waters, sediments, soils and vegetation (EFSA, 2008).

The European Community (EC) has regulated the presence of PAHs in food (European Community 2006). Benzo[*a*]pyrene was previously used as a marker for the occurrence of carcinogenic PAHs in food. In 2008, the Panel on Contaminants in the Food Chain from EFSA (EFSA, 2008) indicated that four PAHs, namely benzo[*a*]pyrene (B[*a*]P), benz[*a*]anthracene (B[*a*]A), benzo[*b*]fluoranthene (B[*b*]F) and chrysene (Chry) are better indicators for the carcinogenic potency of PAHs in food (EFSA, 2008). These are referred to as 4PAH in this report.

Grapes grown in SA vineyards may be exposed to potential sources of PAH contamination, including creosote preservative treatment of wooden posts. In order to successfully analyse whether or not wine made from exposed grapes contains PAHs of significant quantities, a liquid-liquid extraction method (LLE) developed at Stellenbosch University (Panzeri, 2013), using the HPLC-DAD (Diode-Array Detection) was used to analyse the wine samples.

This study compares the HPLC with fluorescence detection (FLD) and UPLC with fluorescence detection (FLD), using the same sample extraction method. This is done to determine which of the two analytical systems is most proficient in detecting low levels of PAHs from wines spiked with a known amount of a PAH mix. Limits of detection (LOD), limits of quantification (LOQ), recovery and precision of the two systems are compared for this purpose.

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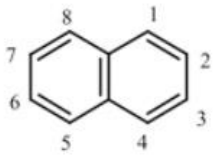
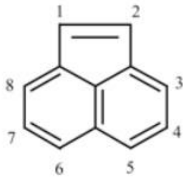
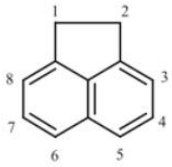
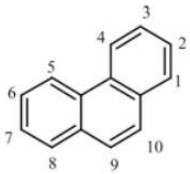
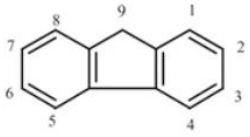
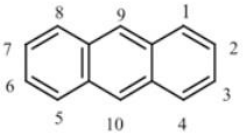
3.2 Materials and Methods

3.2.1 Chemicals, materials and equipment

The 12 PAHs used for this study (PAH Calibration Mix 1x 1 mL, 10 µg/mL in acetonitrile) are listed in Table 1. 2-Ethyl anthracene (2EA) served as the internal standard. The calibration mix and internal standard were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Acetonitrile, cyclohexane (both HPLC grade), and the sodium sulphate anhydrous (Na_2SO_4) were purchased from Merck, Germany. Elix and Milli-Q water purification systems were employed to obtain HPLC grade water (Millipore, Bedford, Massachusetts, USA).

***Table 3.1**: The 12 PAHs and their abbreviations*

Compound	Abbreviation	Structure
Naphthalene	Naph	
Acenaphthylene	Acy	
Acenaphthene	Ace	
Phenanthrene	Phe	
Fluorene	Fluo	
Anthracene	Ant	

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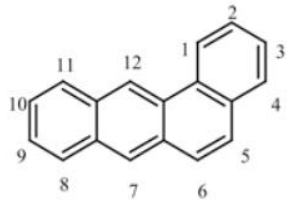
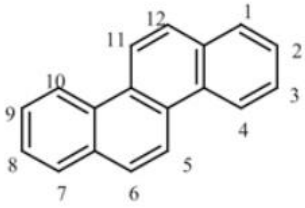
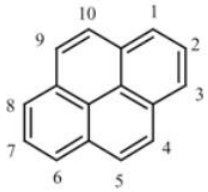
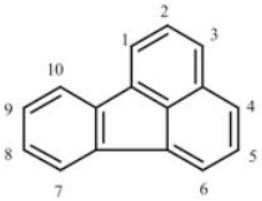
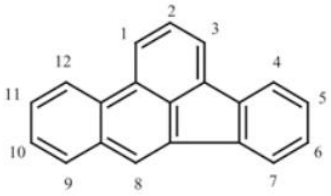
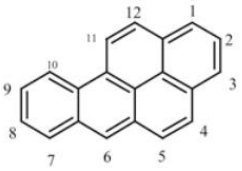
Benzo[*a*]Anthracene	B[*a*]A*	
Chrysene	Chry*	
Pyrene	Pyr	
Fluoranthene	Fla	
Benzo[*b*]fluoranthene	B[*b*]F*	
Benzo[*a*]pyrene	B[*a*]P*	

** The PAHs used as indicator of the carcinogenic potency of PAHs in food (referred to as 4PAH)*

3.2.2 Samples

Three South African red wines from the 2015 vintage were analysed of PAHs to find a clean wine for spiking. The wines were tank samples and had no exposure to barrels. The wines were made from grapes grown in vineyards that did not use creosote posts. These wines were analysed for PAHs

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according to an unpublished in-house laboratory protocol using a Liquid-Liquid Extraction (LLE) method (Panzeri, 2013). They were all found to have no detectable PAH contamination. One of the wines was selected to be spiked at various levels with the PAH mixture. The spiking levels of the PAH mixture were 0.2 ppb, 1 ppb, 2 ppb, 5 ppb, and 10 ppb, plus the control (clean wine). This was done in triplicate. The internal standard was added to each of the spiked samples, as outlined in Section 3.2.3.

3.2.3 Sample preparation (Liquid-liquid extraction)

Sample preparation was done by liquid-liquid extraction (LLE) procedure as described by Panzeri et al (2013). The glassware used for the extraction procedure was cleaned with distilled water and dried in a forced circulation incubator (Labcon, CA) at 60°C. 25 mL of wine was pipetted into 35 mL Pyrex® glass tubes and internal standard (2-ethyl anthracene) was added at a concentration of 40 µg/L. The wine was spiked at the various concentrations. Three mL of cyclohexane (extraction solvent) was added. The Pyrex® glass tubes were then sealed, shaken and placed in the ultrasonicator (Branson Ultrasonic Corporation, Danbury, Connecticut, USA) for 20 minutes. These were shaken every five minutes. After this, the tubes were placed in the freezer at -20°C for 15 minutes in order to reduce internal vapour pressure. The top organic layer was removed using a glass Pasteur pipette (Stargate Scientific, Wilgeheuwel, South Africa) and transferred into a 15 mL glass Pyrex® tube. These tubes were closed with screw top and set aside. The extraction steps were repeated a second time – an additional three mL of cyclohexane was added and the tubes placed in the ultrasonicator for 20 minutes. After 15 minutes in a freezer at -20° Celsius, the organic layer was removed and combined with the organic layer already transferred to the 15 mL glass tube. The tubes were placed in the centrifuge from HERMLE (Labortechnik GmbH, Wehingen, Germany), for five minutes at 4000 rpm. The tubes were taken out and shaken to improve their separation. They were then centrifuged for a further 10 minutes. The clear organic layer was transferred into new 15 mL glass tubes, containing a layer of anhydrous sodium sulphate covering the bottom. These tubes were then vortexed (Scientific Industries Inc., NY, USA) for 30 seconds. The dried organic layer was transferred into new glass tubes, placed in a Techne Dri-block® DB-3D sample concentrator (Techne, Staffordshire, United Kingdom) under a stream of nitrogen at a controlled temperature of 40°C until the sample had evaporated. After evaporation, the residue was re-dissolved in one mL of acetonitrile (Merck, Germany) and vortexed for one minute. The acetonitrile-based mixture was transferred into a one mL syringe (Surgi Plus, Orissa, India) fitted with a 0.45 µm nylon filter (Bonna-Agela Technologies, Wilmington, Delaware, USA**)**. Half of the reconstituted extract was filtered into UPLC glass vials (Stargate Scientific, Wilgeheuwel, South Africa) and sealed; the other half was filtered into HPLC glass vials (Stargate Scientific, Wilgeheuwel, South Africa) and sealed. The samples were stored at 4°C until analysis.

3.2.4 Chromatographic conditions

3.2.4.1 UPLC-FLD analysis

A Waters Acquity UPLC-FLR Detector system with Photodiode Array (PDA) detector was used for PAH analysis. The column was a 2.1 x 150 mm Acquity UPLC HSS T3, 1.8 µm particle size. The

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column temperature was maintained at 45°C during analysis. The instrument is controlled by MassLynx, Version 4.1 software (Waters, Acquity). The mobile phase consisted of water and acetonitrile (ROMIL for Far UV) at a flow rate of 0.350 mL/min. The mobile phase was maintained for 0.05 min. at 35% water. It followed a concave gradient (curve 5), and over 15 minutes increased from 35 to 75% acetonitrile; the gradient remained linear for 5 minutes up to 77%. This was followed by a 2 minute wash step to 100%. The column was re-equilibrated for 3 minutes to starting conditions.

The PDA detector was programmed to take readings from 220 to 800 nm for identification of the compounds and to ensure no co-elution occurs. The excitation wavelength of the FLR detector was set at 260 nm. The emission wavelength was set at zero order, scanning between 210 and 900 for the stronger signal. The injection volume was five µL.

3.2.4.2 HPLC-FLD analysis

Analysis was conducted on a Waters (Milford, Massachusetts, USA) HPLC system equipped with an in-line degasser, 515 HPLC binary pump, 717 plus auto sampler, 2487 dual wave-length absorbance detector and 2475 multi wavelength fluorescence detector. The column used was a Kinetex 2.6 µm Phenyl-Hexyl column (Phenomenex, Toronto, Canada) (150 mm x 4.6 mm; 100 Å). The HPLC uses Empower (2002) software (Waters, Acquity).

The mobile phase consisted of water and acetonitrile (ROMIL, Cambridge, UK). The initial mobile phase was maintained for 2 min at 40% acetonitrile, and over 10 minutes increased from 40 to 62% acetonitrile. It was brought from 62% to 100% in 8 min thereafter. The mobile phase was maintained at 100% for 3 min. The column was re-equilibrated for 12 minutes to starting conditions.

The UV detector was programmed to take readings from 254 to 400 nm for identification of the compounds and to ensure no co-elution occurs. FLD excitation wavelength was set at 260 nm with emission at 360 nm, and 290 nm excitation and 440 nm emission. The flow rate was 1 mL/min and the injection volume was 10 µL. The various compounds elute at different wavelengths (HPLC-FLD), as seen in table 2.

***Table 3.2**: The wavelengths of the various PAHs detected by the HPLC-FLD*

λ	**Compounds**
230 nm	Pyr
254 nm	Acy; Ant
260/352	Ace; Naph; Flu; Phe; 2EA; Chry
290/440	Fla; B[*a*]A; B[*b*]F; B[*a*]P

3.2.5 Validation Data

3.2.5.1 Linearity, selectivity and specificity

The wine samples with PAH concentrations below LOD were considered clean and used for further analysis. The clean wines were spiked with different amounts of the PAH mixture (0, 0.2, 1.0, 2.0,

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5.0 and 10 ppb) and analysed as described above. Quantification of the compounds was done by integrating the area of the peaks and dividing it by the area of the internal standard.

Linearity of calibration curves was assessed by calculating the regression coefficient.

Selectivity of the method was verified by comparing the eluted compounds with the retention times of the standards in the PAH mixture that was added to the wine sample. Triplicate clean wine samples were individually extracted and injected. Specificity of separation was determined by comparing chromatograms from red and white wine extractions, to determine if the peaks were the same and that there were no interferences.

3.5.2.2 Limits of detection (LOD), limits of quantification (LOQ), recovery and precision

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration curve at concentrations from 0.2 to 10 ppb for both the UPLC and HPLC. The LOD was calculated based on signal-to-noise ratio of 1:3 and the LOQ was based on a signal-to-noise ratio of 1:10.

The percentage PAH yield recovered was calculated from the calibration curve at concentrations from 0.2 to 10 ppb for both the UPLC and HPLC.

The precision of the two methods was evaluated by spiking the clean wine with known amounts of the PAH mixture and measuring the relative standard deviation of the PAH yield recovered (% RSD).

3.2.6 Statistical analysis

Statistical analyses were performed using the Statistica 9.0 (StatSoft, Inc., 2000) program. Samples were extracted in triplicate.

3.3 Results and Discussion

3.3.1 Retention times, selectivity and specificity

Wine samples were extracted according to the LLE method, and analysed on both the HPLC and UPLC. The retention time (RT) for the elution of the PAHs is recorded in Table 3.3. The total time of analysis on the HPLC was 29.48 minutes and 17.90 minutes for the UPLC. These times include the 12 minute re-equilibration required by the HPLC and the 2 minute re-equilibration required by the UPLC.

Figures 3.1 and 3.2 show an example of typical chromatograms obtained during separation of the 12 PAHs by HPLC and UPLC, respectively. To demonstrate the separation of the 12 compounds, a PAH mixture of 50 ppb was used.

Selectivity: The chromatograms of the unspiked wine sample did not show any peaks at the respective retention times, indicating that the wine does not contain any of the 12 PAHs above the limit of detection. Once the wine was spiked with the 12 PAH mixture, the retention times of the compounds corresponded to those of the pure standards.

Specificity was validated by comparing chromatograms from red and white wines that had been spiked. The wine matrix did not interfere with the peaks of the PAH standard mixture.

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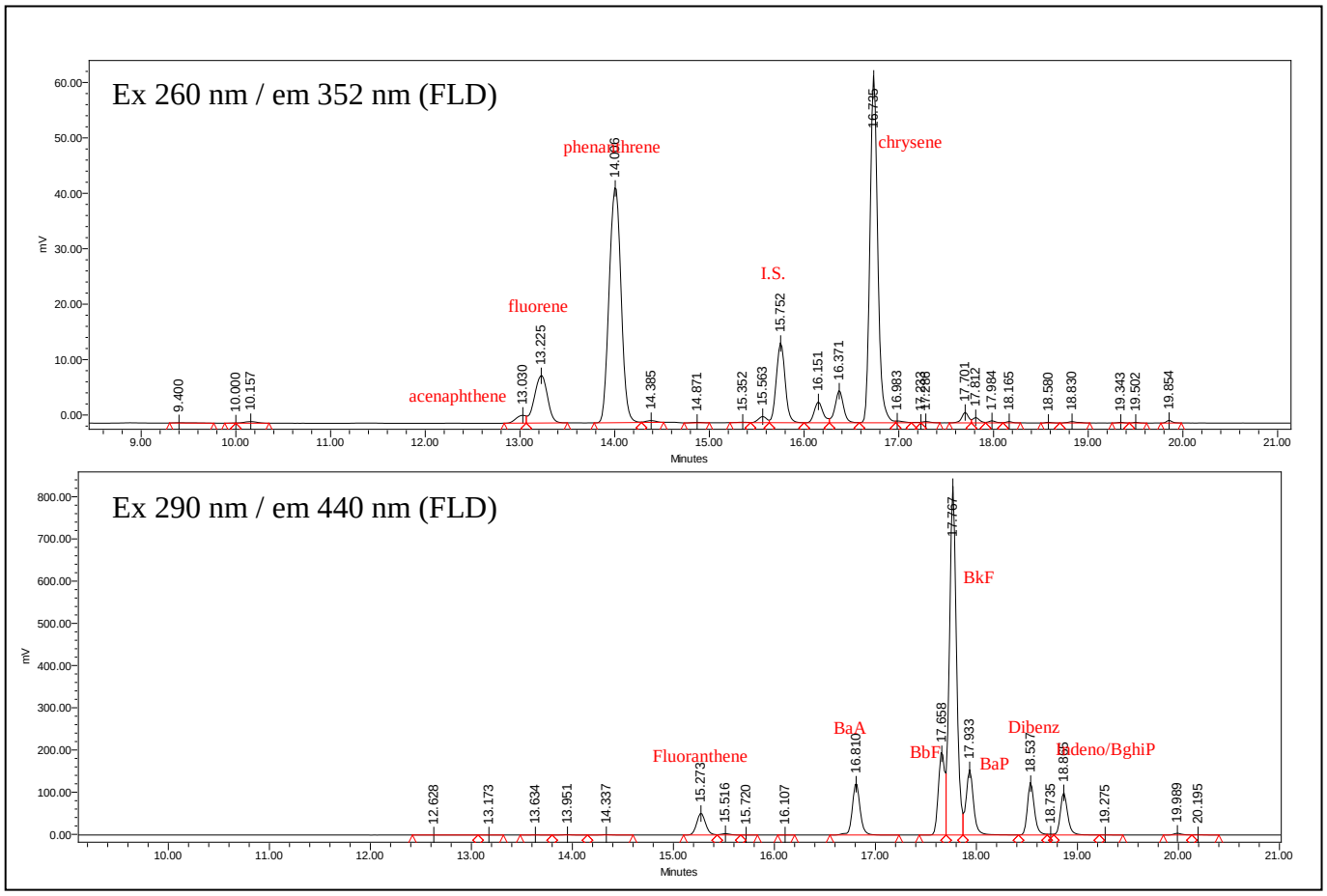

Figure 3.1: HPLC Chromatograms of red wine spiked with 12 PAHs at 50 ppb. The wavelengths monitored were 260/352 nm for Ace, Fluo, Phe, Chry, 2EA; 290/440 nm for Fla, B[*a*]A, B[*b*]F, B[*a*]P and Benzo[g,h,i]perylene (B[*ghi*]P).

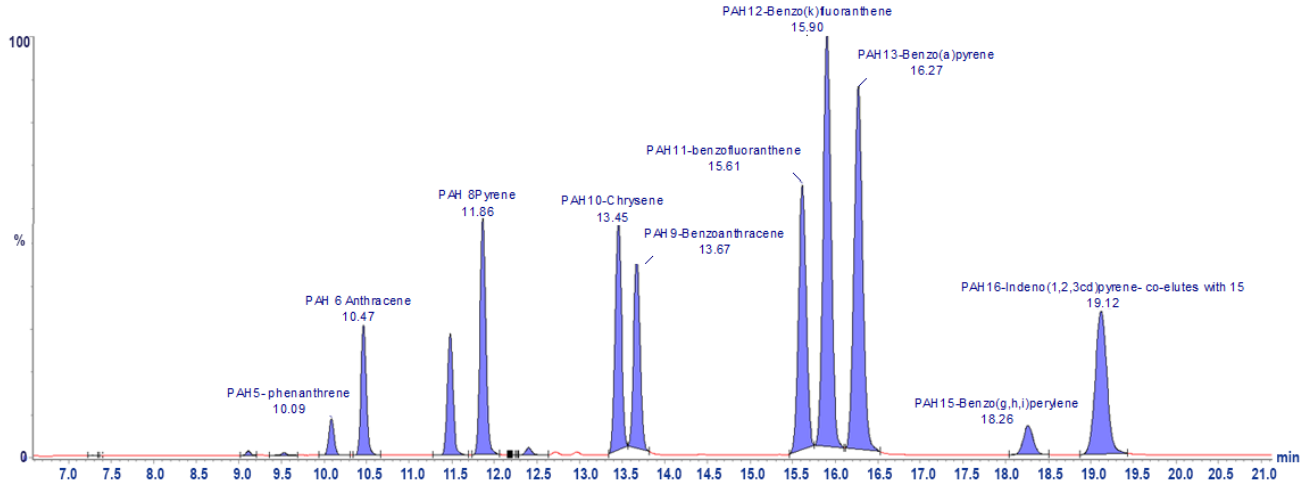

Figure 3.2: UPLC chromatogram of red wine spiked at 50 ppb (excitation of260 nm and emission of 210 – 900 nm).

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Table 3.3: *Comparison of retention times (RT) of the 12 PAHs for the HPLC and UPLC methods (of the spiked samples).*

Compound	**RT for HPLC (min)**	**RT for UPLC (min)**
Naph	9.82	7.21
Acy	11.34	9.05
Ace	12.69	9.21
Flu	12.90	9.50
Phe	13.67	10.09
Ant	14.10	10.46
Fla	15.14	11.50
Pyr	15.20	11.86
IS (2EA)	16,70	12,40
Chry	16.72	13.45
B[*a*]A	16.61	13.67
B[*b*]F	16.61	15.61
B[*a*]P	17.48	15.90

3.3.2 Validation data

3.3.2.1 Linearity

Calibration was done at 0.2, 1.0, 2.0, 5.0 and 10 ppb for the clean wine. During validation testing naphthalene, acenaphthylene, acenaphthene and fluorene showed non-reproducible behaviour. This can be attributed to the semi-volatile nature of these four compounds and explained by partial evaporation during the drying step of sample preparation. Because of this, no values are indicated for these compounds in Table 3.4 and 3.5. The correlation coefficients were higher than 0.985 for the HPLC. The correlation coefficients were higher than 0.985 for Fla, Pyr, Chry, B[*a*]A, B[*b*]F and B[*a*]P for the UPLC. These results showed a satisfactory linearity within the concentration range investigated for both methods, for most of the PAHs.

Table 3.4: Comparison of the correlation coefficient for the PAHs

PAH	UPLC	HPLC
	R^2	R^2
Naph		
Acy		
Ace		
Flu		
Phe	0.871	0.997
Ant	0.872	0.998
Fla	0.991	0.976
Pyr	0.991	0.974
Chry	0.987	0.975
B[a]A	0.988	0.980
B[b]F	0.988	0.980
B[a]P	0.989	0.984

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3.3.2.2 Limits of detection (LOD), limits of quantification (LOQ), recovery and precision

The LOD and the LOQ for both the HPLC and UPLC conditions are shown in Table 3.5. The performance criteria for methods of analysis are an LOD of at least 0.3 ppb and an LOQ of at least 0.9 ppb and percentage recovery between 50 and 120%, as required for the analysis for B[*a*]P (adapted from 2005/10/EC). The LOQ and LOD for both the HPLC and the UPLC are within the recommended limits for most compounds. The two compounds that do not fall within the recommended range include Anthracene (HPLC) and B[*b*]F which is just above the limit of 0.9 ppb set for the LOQ on the UPLC.

Table 3.5: *Results obtained for the HPLC and UPLC instruments*

PAH	HPLC		UPLC	
	LOQ (ppb)	LOD (ppb)	LOQ (ppb)	LOD (ppb)
Naph				
Acy				
Ace				
Flu				
Phe	0.14	0.48	0.08	0.27
Ant	0.45	1.51	0.23	0.78
Fla	0.17	0.56	0.17	0.56
Pyr	0.20	0.68	0.24	0.81
Chry	0.19	0.63	0.07	0.24
B[a]A	0.05	0.16	0.22	0.72
B[b]F	0.22	0.74	0.27	0.91
B[a]P	0.18	0.62	0.21	0.69

The compounds were quantified based on the recovery of PAHs from spiked samples. Panzeri (2013) noted that the recovery values of the LLE method, as well as the LOD and LOQ for the HPLC method, complied with European regulations. The sampling methods and requirements for the methods of analysis for the official control of the levels of B[*a*]P in foodstuffs also comply with European regulations (The Commission of the European Communities, 2005). In this study, the recovery percentages were calculated for the spiked levels for both the HPLC and UPLC analyses (Table 3.6) and range from 81 to 120%. In all cases, the recovery was within the recommended guidelines according to European regulations (The Commission of the European Communities, 2005), of 50 to 120%.

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***Table 3.6**: Recovery percentages of the 4PAH compounds at a concentration of 0.2, 1, 2, 5 and 10ppb for the HPLC and UPLC systems.*

	HPLC					UPLC				
PAH	0.2 ppb	1 ppb	2 ppb	5 ppb	10 ppb	0.2 ppb	1 ppb	2 ppb	5 ppb	10 ppb
Naph										
Acy										
Ace										
Flu										
Phe	97.93	129.95	102.39	95.86	100.12	98.69	100.80	98.38	101.31	102.03
Ant	96.08	122.22	100.44	84.65	100.27	102.08	99.50	96.52	99.91	101.99
Pyr	111.94	95.81	100.62	121.05	94.91	97.98	98.95	96.41	99.62	101.61
Chry	110.00	81.56	110.07	100.66	94.82	91.78	99.66	99.15	105.50	93.79
B[a]A	119.25	81.31	109.08	103.07	95.39	98.50	95.89	98.29	97.00	100.77
B[b]F	120.57	82.32	110.71	103.01	95.42	98.50	95.89	98.29	98.43	99.64
B[a]P	116.22	109.54	93.00	103.17	104.08	99.55	95.19	94.65	99.00	100.25

Table 3.7 shows the Standard Deviations (SDs) and Relative Standard Deviations (RSDs) between the recovery percentages, which range from 3.41 to 160.01%, indicating large variability between the extraction repeats. The RSD of the 4PAHs (Chry, B[*a*]A, B[*b*]F and B[*a*]P) is generally lower than that of the other PAHs listed, ranging from 4.00 to 33.91%. Generally, the standard deviations are fairly high for both the HPLC and the UPLC, indicating variability in the extraction method.

Precision of the instrument was tested by analysing pure calibration standards at 50 ppb, 6 times, and comparing the relative standard deviation of the recovery percentage. The RSDs for compounds were below 2.3%, indicating that the precision of the instrument is within an acceptable range.

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Table 3.7: *Standard deviation (SD) and Relative Standard Deviation (%RSD) of the recovery percentage of PAHs for the spiked wines, analysed using the HPLC and UPLC.*

PAH	Method	HPLC					UPLC				
	Ppb	0,2	1	2	5	10	0,2	1	2	5	10
Phe	SD (ppb)	0,32	0,08	0,52	1,65	3,48	0,08	0,13	0,37	1,34	3,07
	%RSD	160,01	8,33	26,01	33,06	34,83	40,17	13,41	18,47	26,85	30,67
Ant	SD (ppb)	0,08	0,08	0,40	1,36	3,11	0,08	0,05	0,35	1,17	3,00
	%RSD	41,50	8,40	20,09	27,27	31,13	41,67	4,72	17,25	23,40	30,00
Fla	SD (ppb)	0,04	0,12	0,07	0,41	0,69	0,01	0,20	0,11	0,40	1,04
	%RSD	21,82	11,76	3,49	8,23	6,90	3,41	19,88	5,40	8,05	10,35
Pyr	SD (ppb)	0,25	0,41	0,26	0,59	0,55	0,03	0,17	0,12	0,42	1,04
	%RSD	126,30	41,00	13,17	11,83	5,53	13,44	17,16	6,18	8,40	10,37
Chry	SD (ppb)	0,03	0,16	0,23	0,76	0,76	0,03	0,10	0,19	0,70	0,92
	%RSD	13,61	16,08	11,29	15,17	7,61	13,47	10,43	9,28	14,08	9,23
B[a]A	SD (ppb)	0,02	0,16	0,22	0,77	0,51	0,02	0,17	0,11	0,71	0,94
	%RSD	11,76	15,67	11,02	15,49	5,09	12,41	17,22	5,39	14,14	9,38
B[b]F	SD (ppb)	0,03	0,16	0,24	0,89	0,80	0,02	0,22	0,11	0,88	0,55
	%RSD	13,42	16,07	11,87	17,88	8,02	11,71	21,69	5,69	17,64	5,50
B[a]P	SD (ppb)	0,02	0,07	0,08	0,53	0,45	0,07	0,21	0,11	0,88	0,66
	%RSD	10,12	6,99	4,00	10,54	4,54	33,91	20,97	5,53	17,66	6,57

3.3.3 Comparison of results from the HPLC and UPLC methods

The correlation between the results from the HPLC and the UPLC, as well as the recovery levels of the 4PAHs (Chry, B[*a*]A, B[*b*]F and B[*a*]P) are compared in figures 3.3, 3.4, 3.5 and 3.6. These four PAHs were selected to represent the recoveries as they are used as indicators of the carcinogenic potency of PAHs in food (EFSA, 2008).

These figures show a comparison between the performance of the HPLC and UPLC. The R^2 values are 0.99, 0.98, 0.98 and 0.97, respectively. These strong correlations indicate that analysis of the four PAHs is consistent and similar between the HPLC and UPLC.

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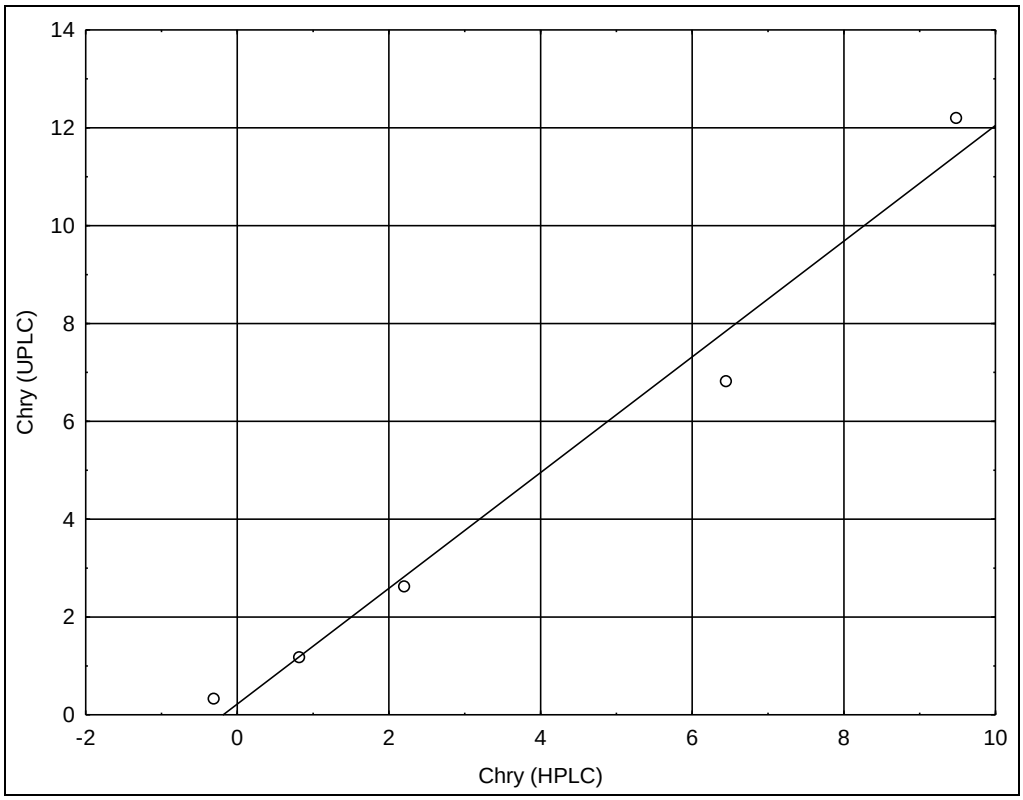

Figure 3.3: A comparison of the Chyr ppb value after spiking wine at 0.2, 1, 2, 5, 10ppb and analysing with the UPLC and the HPLC where $R^2 = 0.98$ and $y = 1.183x + 0.222$

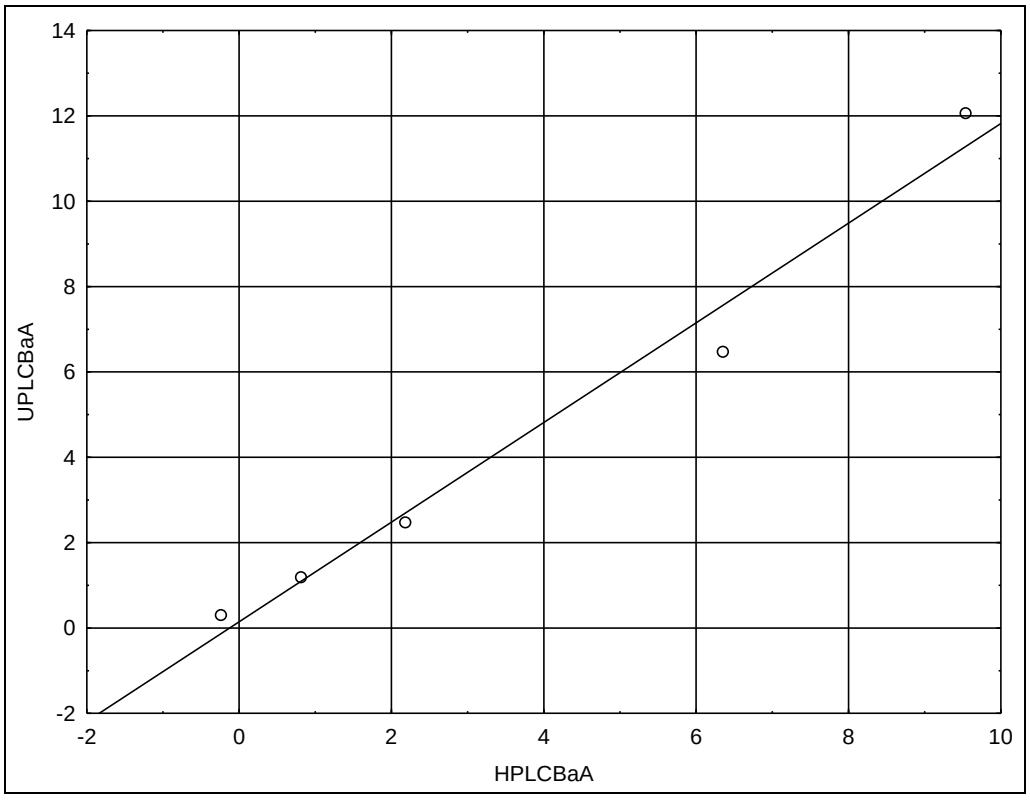

Figure 3.4: A comparison of the B[a]A ppb value after spiking wine at 0.2, 1, 2, 5, 10ppb and analysing with the UPLC and the HPLC where $R^2 = 0.98$ and $y = 1.168x + 0.146$

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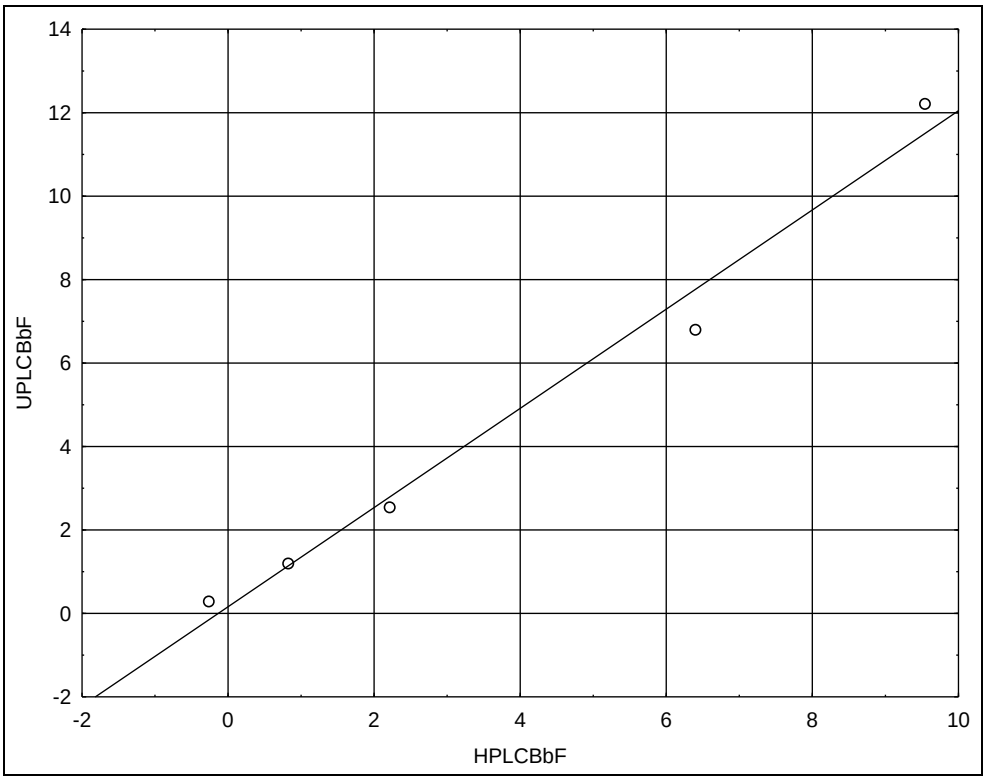

Figure 3.5: A comparison of the B[b]F ppb value after spiking wine at 0.2, 1, 2, 5, 10ppb and analysing with the UPLC and the HPLC where $R^2 = 0.98$ and $y = 1.189x + 0.160$

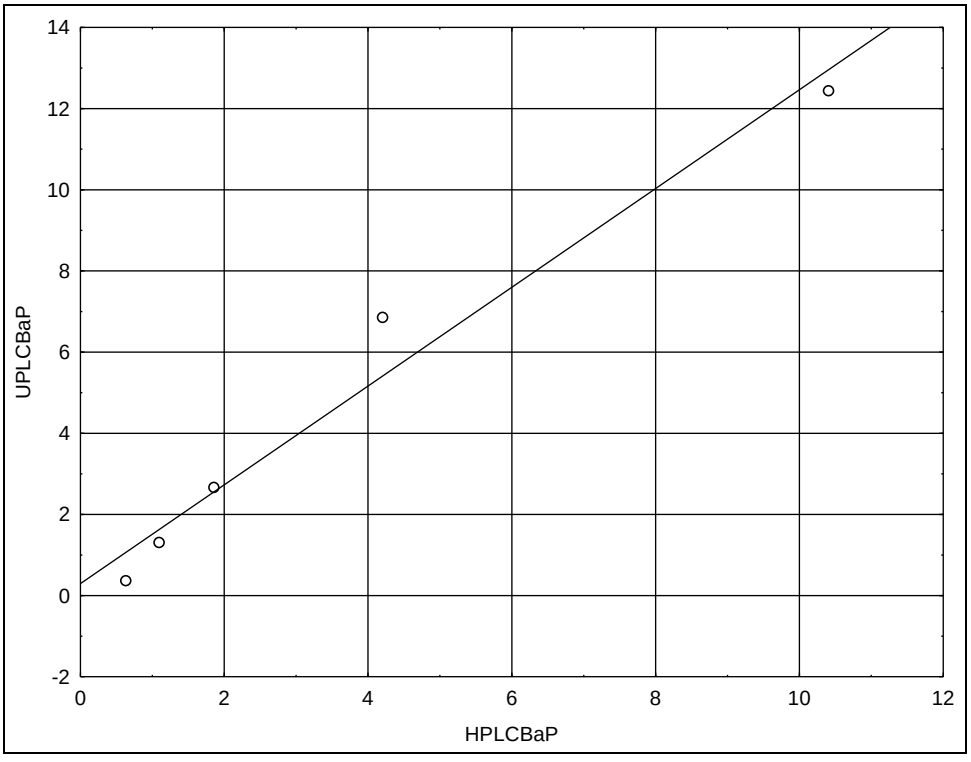

Figure 3.6: A comparison of the B[a]P ppb value after spiking wine at 0.2, 1, 2, 5, 10ppb and analysing with the UPLC and the HPLC where $R^2 = 0.97$ and $y = 1.217x + 0.295$

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3.4 Conclusions

The HPLC and UPLC methods for the determination of PAHs in wine were compared. Both methods showed a satisfactory linearity within the concentration range investigated over the entire calibration ranges. Both methods successfully detected most of the 12 PAHs in question at low levels. Due to the volatile or partially volatile nature of the smaller PAHs with only two or three rings (Naphthalene, Acenaphthene, Acenaphthylene, Phenanthrene, Fluorene and Anthracene), they are not as accurately detected as the HMW PAHs.

The LOQ and LOD of both methods fell within the recommended range for all the PAHs except two (Anthracene and B[*b*]F). Both the UPLC and HPLC were able to detect and quantify the PAHs to acceptable levels.

The performances of the UPLC and HPLC were determined and compared. There was a strong correlation between the two methods of analysis, indicating that the analysis of the PAHs was consistent and similar between the HPLC and UPLC.

It is recommended that further research is conducted to investigate the precision of the extraction method.

The length of time required for the analysis per sample was 29.48 minutes for the HPLC and 17.90 minutes for the UPLC. Both methods are time consuming, with the UPLC being one third faster than the HPLC.

3.5 References

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Chapter 4

Determination of Polycyclic Aromatic Hydrocarbons in wine using UPLC-FLD

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CHAPTER IV: DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN WINE USING UPLC-FLD

Abstract

This study describes the occurrence of polycyclic aromatic hydrocarbons (PAHs) in a variety of red and white wines, via the monitoring of 12 priority PAHs, with a focus on the four PAHs that have been chosen as indicators for the occurrence of PAHs (4PAH) in food by the European Food Safety Agency: Chry, B(*a*)A, B(*b*)F and B(*a*)P. Of the latter group, chrysene was detected at the highest levels in the wines, ranging from 0.03 to 0.61 ppb. The sum of the 4PAHs ranged from 0.05 to 1.52 ppb. The B[*a*]P levels in the wines ranged from 0.01 to 0.39 ppb. PAH levels in the majority of the wines were below 0.50 ppb. The PAH concentrations in wine are very low relative to the legal limits set for smoked and char-broiled foodstuffs.

4.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds consisting of two or more fused aromatic rings. While not yet officially classified as persistent organic pollutants (POPs) under the Stockholm Convention, these organic contaminants are resistant to degradation, can remain in the environment for long periods and have the potential to cause adverse environmental effects. PAHs occur in all parts of the environment: atmosphere, inland and sea waters, sediments, soils and vegetation.

The wine industry is very important to the South African Economy, contributing R36.1 billion gross domestic product (GDP) to the regional economy and employing 300000 people both directly and indirectly in 2013. In 2014, South Africa ranks as number seven in the world in overall volume production of wine and produced 4.2% of the world's wine (SAWIS, 2015).

Exports of natural (*i.e.* non-fortified) packaged wines in 2014 reached 173.4 million litres, and the total exports of wine were 422.7 million litres.

The South African wine industry continuously tries to align itself with international trends and expectations. Polycyclic aromatic hydrocarbons (PAHs) have been the subject of on-going scrutiny and escalating legislation, particularly in the European Union and the United States, because of their mutagenic and carcinogenic properties.

Wine grapes grown in South African vineyards may be exposed to various sources of PAHs. These sources include the use of creosote posts in vineyards, bush fires and vehicle emissions. PAHs are lipophilic and may contaminate wine directly by adhering to the waxy cuticle of the grape skin. Studies are being conducted to determine if contamination can occur indirectly by the uptake of PAHs through the soil by the roots. The aim of this study is to determine if exposure to PAHs has resulted in the presence of PAHs in the wine, and at which levels these PAHs may be present.

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Following the scientific opinion of the European Food Safety Authority (EFSA) in 2007, the European Commission fixed limits for benzo(*a*)pyrene and the sum of 4PAH in different foodstuffs in the amendment 835/2011 of the regulation 1881/ 2006. 4PAH include chrysene (Chry), benzo(*a*)anthracene (B[*a*]A), benzo(*b)*fluoranthene (B[*b*]F) and benzo(*a*)pyrene (B[*a*]P), and serve as an indicator for the occurrence of PAHs in food (EFSA, 2008). Wine is not covered by this regulation. However, the PAH limits for drinking water, set by the Commission Regulation 98/83/EC, limits the amount of B[*a*]P to 0.01 ppb, and the sum of 4 PAHs to 0.1 ppb. The Agency for Toxic Substances and Disease Registry (ATSDR, 1995) set the limit of B[*a*]P in water to 0,2 ppb. The European Union set the B[*a*]P legal limit of 1 ppb for all dietary food (European Directive 2006/125/EC). The Czech Republic is the only country with a 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) in wine, of 0.5 ppb (Wenzl *et al*, 2006). The suggested maximum limits of B[*a*]P allowed in alcoholic beverages are 2.0 ppb (Van Zyl, 2013).

The method of extraction and analysis used in this study was developed at Stellenbosch University (Panzeri, 2013). The analyses of the extracts were done by an ultrahigh pressure liquid chromatography fluorescence detector system (UPLC-FLD), with Photodiode Array (PDA) detector.

4.2 Materials and methods

4.2.1 Chemicals, materials and equipment

The PAH standards used for the calibration are listed in Table 1. Refer to Chapter 3 for the list of chemicals, materials and equipment used.

Table 4.1*: The 12 PAHs and abbreviations*

Compound	**Abbreviation**
Naphthalene	Naph
Acenaphthylene	Acy
Acenaphthene	Ace
Phenanthrene	Phe
Fluorene	Fluo
Anthracene	Ant
Benzo[*a*]Anthracene	B[*a*]A*
Chrysene	Chry*
Pyrene	Pyr
Fluoranthene	Fla
Benzo[*b*]fluoranthene	B[*b*]F*
Benzo[*a*]pyrene	B[*a*]P*

** The PAHs that serve as indicator of the carcinogenic potency of PAHs in food (referred to as 4PAH)*

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4.2.2 Samples

A range of South African and international wines were selected for this study. Some of the wines were selected randomly, while others were selected based on the exposure the grapes may have had to PAH sources. Some were selected because of their burnt rubber aroma, an aroma that may be associated with exposure to creosote posts (Panzeri 2013). Of the 57 South African wines, 32 were from Stellenbosch region, eight from Paarl, four from the Swartland and Roberton regions, and one each from the Breede River, Carlitzdorp, Constantia, Durbanville, Franschhoek, Northern Cape, Wellington and Worcester areas. The wines were a mix of red and white wines, as well as two Sherries and three port-style wines. Red blends made up 17 of the wines, together with 16 Shiraz, seven Cabernet Sauvignon, two Merlot, one Pinot Noir and a Ruby Cabernet. The white wine selection comprised two Chenin blanc, two Sauvignon blanc, one Chardonnay and a white blend. The vintages ranged from 1980 to 2012, with the majority of the wines coming from 2007 (6), 2008 (8), 2009 (16), 2010 (7) and 2011 (6). It is important to note that 2008 and 2009 were vintages that experienced a large number of bush fires in South Africa. Of the 57 wines, at least 25 came from vineyards that use creosote posts. Four of the wines were made from bush vines. A large number of the selected wines had been fermented and/or aged in oak barrels.

The international wines were from Germany (4), Spain (4), Portugal (4), Italy (1) and Australia (1). They consisted of five red wines, six white wines and two ports.

The wine samples were stored at 4°C in 200 mL amber bottles until extraction.

4.2.3 Sample preparation

Wines were analysed for PAHs according to a liquid-liquid Extraction (LLE) method (Panzeri, 2013).

4.2.4 Chromatographic conditions

A Waters Acquity UPLC-FLR Detector system was used for PAH analysis of these wines. Refer to Chapter 3 for the Chromatographic conditions.

The resulting chromatograms, and the compounds monitored at each wavelength, can be seen in Figure 2, showing that the PAHs elute clearly and good levels of separation were achieved.

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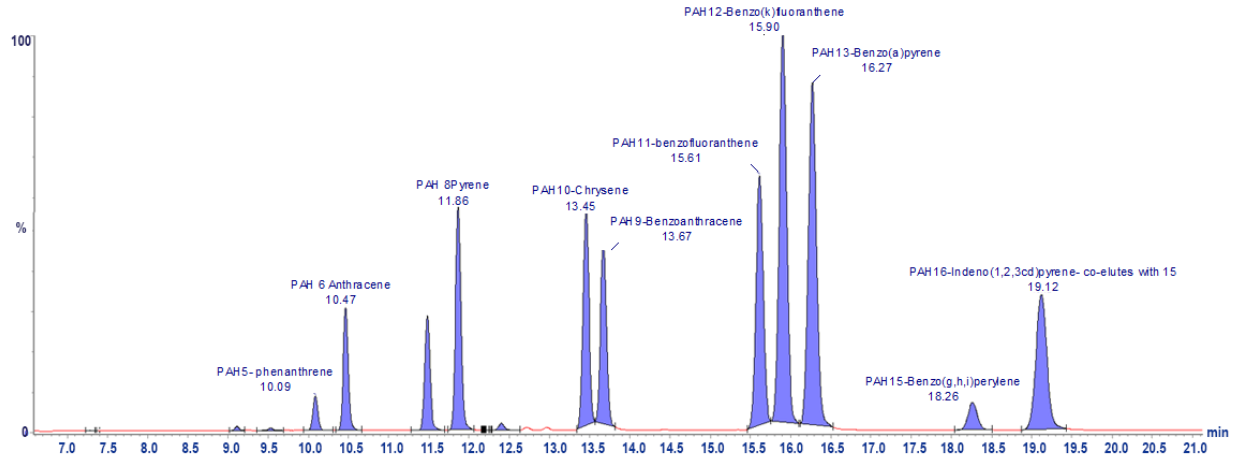

Figure 4.1: UPLC chromatogram of red wine spiked at 50 ppb (excitation of 260 nm and emission of 210 - 900).

4.2.5 Linearity, selectivity and specificity

A clean wine was spiked with different amounts of PAHs (5, 10, 25, 50 and 100 ppb) and analysed. Quantification of the compounds was done by integrating the area of the peaks and dividing it by the area of the internal standard. Linearity of calibration curves was assessed by calculating the regression coefficient.

Selectivity and specificity of the method was verified as described in Chapter 3.

4.2.6 Limits of detection (LOD), limits of quantification (LOQ), recovery and precision

The limits of detection (LOD) were calculated from the calibration curve at concentrations from 5 to 100 ppb. The LOD was calculated based on signal-to-noise ratio of 1:3 and the LOQ was based on a signal-to-noise ratio of 1:10. A 'clean' wine (previously analysed to ensure it was free of PAHs) was selected. A calibration curve was set up by spiking the wines with PAH standards. This was done in triplicate. The spiking levels were 5, 10, 25, 50 and 100 ppb, plus a blank (clean wine). Once the wine was spiked to the five different levels, it was extracted in triplicate, using the liquid-liquid extraction (LLE) method and analysed using the UPLC-FLD.

The percentage recovery of the PAHs was calculated from the calibration curve at concentrations. The precision of the method was determined by calculating the relative standard deviations (%RSD) between the repeats of the extractions.

4.3 Results and discussion

4.3.1 Linearity, Limits of detection (LOD), limits of quantification (LOQ), recovery and precision

The retention time of each compound is listed in Table 4.2. The R^2 values are close to 1 (except for the first four compounds listed), which indicates good linearity in the calibration sets. The limit of detection (LOD) is listed for all the compounds except for naphthalene, acenaphthylene, acenaphthene and fluorene. As described in Chapter 3, these four compounds are volatile or semi-

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volatile, making quantitation unreliable. The LOD levels for the rest of the PAHs falls within the required range of less than 0.3 ppb, complying with European regulations, in particular with regard to Benz[*a*]pyrene (The Commission of the European Communities 2005). The concentrations of many of the PAHs found in the sample wines were above the LOD and the LOQ. The range of PAHs in the sample wines is below the levels set up in the calibration curve. This was an unexpected result. Preliminary work done prior to this study indicated that the expected PAH levels found in some South African wines were between 10 and 200 ppb, hence the range of the calibration curve selected. The calibration curves were compared to the calibration curves in chapter 3 (0.2 – 10 ppb) for the different components, and the curves were found to be similar, with an R^2 of 0.95. Figure 4.2, 4.3, 4.4 and 4.5 compare the two calibration curves for Chry, B[a]A, B[b]F and B[a]P. Because of the good correlation between the two calibration series, the values obtained using the calibration curve for 10 to 200 ppb were used.

For future work, it is still recommended that the calibration curve is in the range of 0.2 – 20 ppb, and the samples are extracted and analysed using this curve, to ensure accuracy of the results.

Table 4.2: Retention time (RT) the calibration curve, the R^2 value, the limit of detection (LOD) and the range found in wine samples. The 4PAHs that serve as indicator of the carcinogenic potency of PAHs in food include Chry, B[a]A, B[b]F and B[a]P.

Compound	**RT (min)**	**Calibration curve**	**R^2**	**LOD**	**LOQ**	**Range in sample wines (ppb)**
Naph						
Acy						
Ace						
Flu						
Phe	11.40	y = 0.016x + 0.06	0.98	0.03	0.01	0.3 - 5.84
Ant	12.53	y = 0.037x + 0.09	0.99	0.03	0.01	0.3 - 1.58
Pyr	12.93	y = 0.06x + 0.02	1.00	0.02	0.07	0.2 - 2.97
Chry	14.69	y = 0.06x + 0.03	1.00	0.03	0.01	0.3 - 0.99
B[*a*]A	14.93	y= 0.05x + 0.01	1.00	0.03	0.01	nd - 0.51
B[*b*]F	16.92	y = 0.05x + 0.004	1.00	0.02	0.07	nd - 0.57
B[*a*]P	17.53	y= 0.12x + 0.02	1.00	0.03	0.01	nd - 0.58

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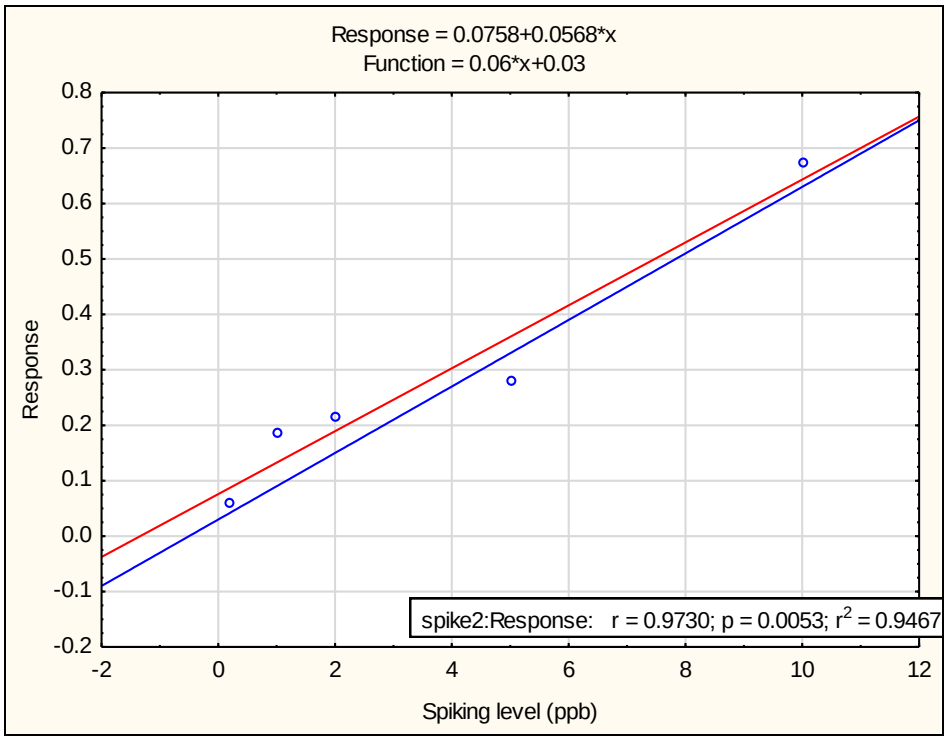

Figure 4.2: Calibration curve of Chrysene from the calibration series: 10 – 200 ppb (blue), compared to the calibration series: 0.2 – 10 ppb (red)

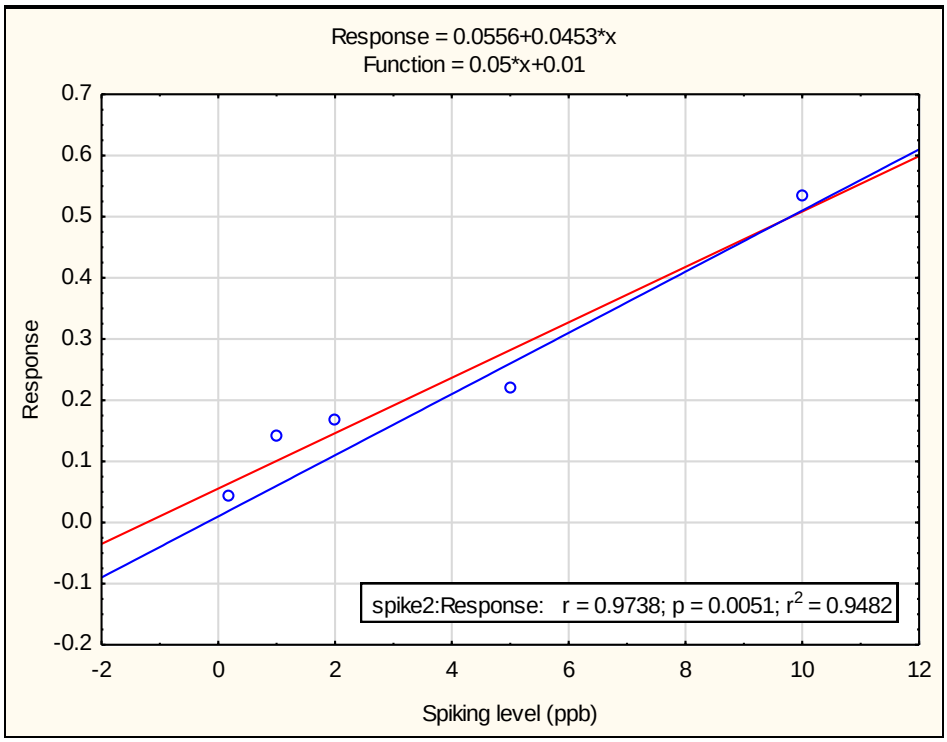

Figure 4.3: Calibration curve of B[*a*]A from the calibration series: 10 – 200 ppb (blue), compared to the calibration series: 0.2 – 10 ppb (red)

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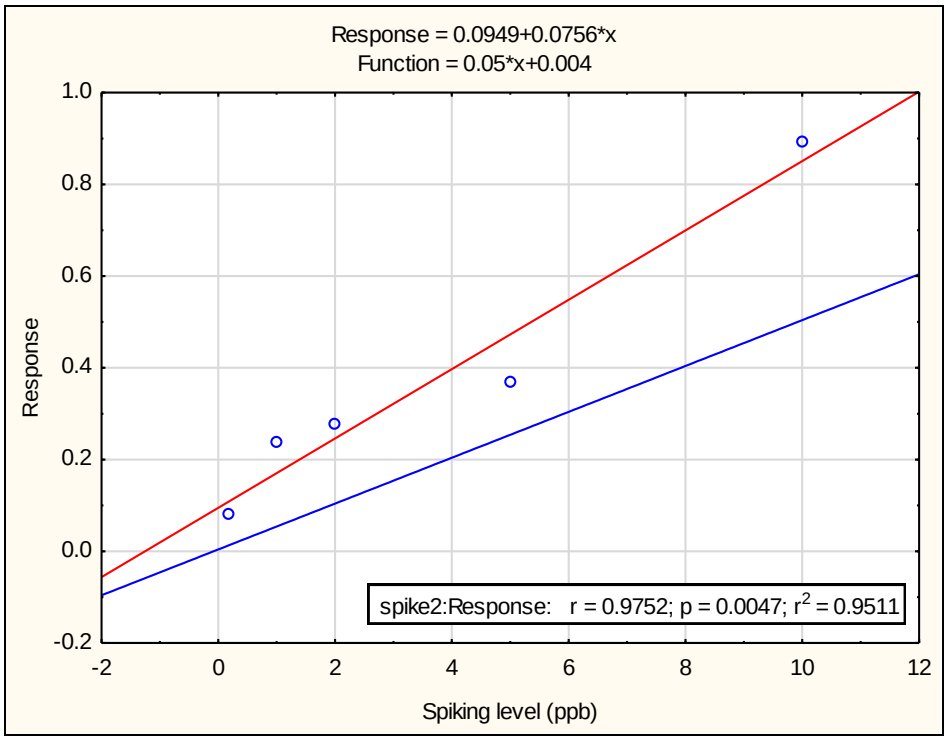

Figure 4.4: Calibration curve of B[*b*]F from the calibration series: 10 – 200 ppb (blue), compared to the calibration series: 0.2 – 10 ppb (red)

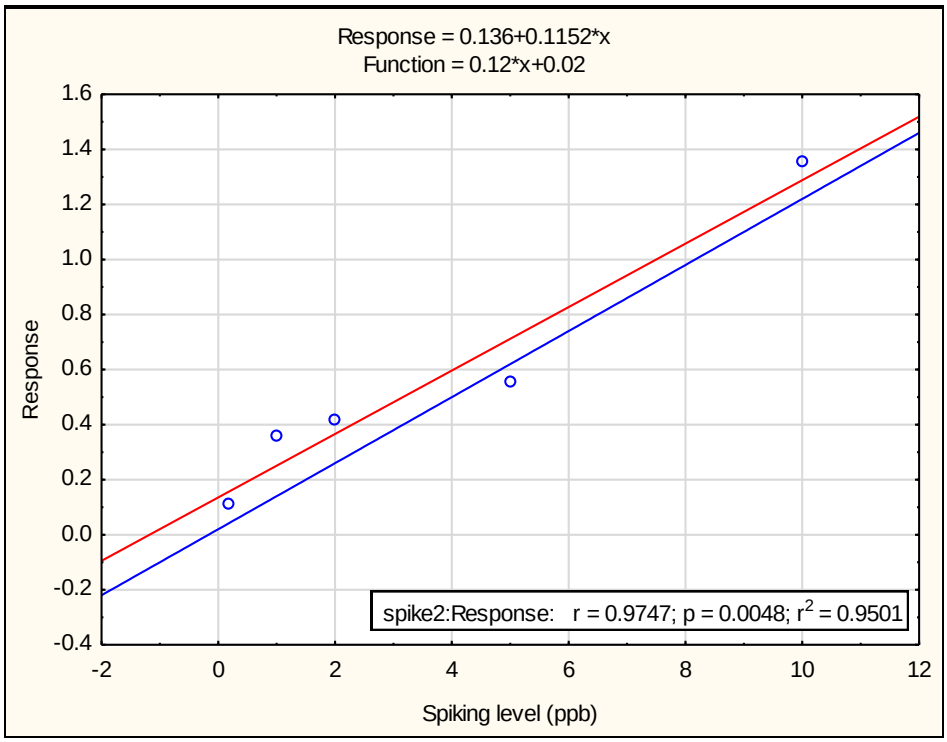

Figure 4.5: Calibration curve of B[*a*]P from the calibration series: 10 – 200 ppb (blue), compared to the calibration series: 0.2 – 10 ppb (red)

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The linearity of the calibration was variable for the smaller PAHs of low molecular weight (LMW) (naphthalene, acenaphthylene, acenaphthene and fluorene). The LMW PAHs are semi-volatile, and less easy to quantify accurately due to evaporation during the extraction procedure. During the development of the liquid-liquid extraction method by Panzeri (2013), similar findings were observed for these four compounds. The R^2 values for the HMW PAHs are close to 1, showing good linearity.

The recoveries of the PAHs, using the methods described in Chapter 3, are within the recommended range of 50-120%. In a similar study done on the analysis of PAHs in fruit and vegetables in Brazil (Carmago, 2003), the validation data was all found to be within the limits of what are set by the Commission of the European Communities (2005) for Benzo[*a*]pyrene. The RSD values reported ranged from 6 to 21% and were deemed satisfactory for the determination of PAHs at the µg/kg level.

Yoo *et al.* (2014) compared two extraction methods for the analysis of PAHs in seafood, and was also able to achieve excellent precision of less than 6%.

The standard deviations (SD) can be seen in Table 4.4 for South African wines and in Table 4.5 for international wines. The variation between the repeats is generally high, as indicated by the SDs, however, all the criteria set by the Commission of the European Communities (2005) for Benzo[*a*]pyrene have been met in this study, except for the repeatability of the extraction method. However, despite being variable, the PAH levels of the individual wines remain within the 5ng/kg suggested limits for B[*a*]P recommended for drinking water (AFFSA, 2006).

Overall, the PAHs levels found in both the South African and international wines were relatively low (Tables 4.4 and 4.5). The PAH concentration for the sum of the 4PAH ranged from 0.05 to 1.52 ppb, with 68% of the wines having 4PAH levels below 0.50 ppb (as prescribed by the Czech Republic legislation). No wines had B[*a*]P levels over 1.00 ppm, the legal limit for all dietary food according to the European Union. This is below the maximum allowed level in drinking water for the sum of the four PAHs and below the suggested limit of 2.00 ppb for alcoholic beverages (AFFSA, 2006).

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Table 4.4: *The level of 4PAH in South African wines (n=54), as well as the standard deviations (SD)*

Wine code	Chry		B[*a*]A		B[*b*]F		B[*a*]P		∑4PAH
	Conc (ppb)	SD	Conc (ppb)	SD	Conc (ppb)	SD	Conc (ppb)	SD	Conc (ppb)
A	0.28	0.01	nd	0.01	0.13	0.02	nd	0.02	0.44
B	0.30	0.06	0.04	0.06	0.18	0.10	0.09	0.14	0.61
C	0.31	0.03	0.04	0.04	0.20	0.03	0.06	0.03	0.60
D	0.29	0.02	0.03	0.01	0.19	0.01	0.05	0.02	0.57
E	0.61	0.33	0.05	0.03	0.40	0.16	0.08	0.05	1.14
F	0.26	0.03	0.04	0.03	0.18	0.02	0.04	0.02	0.52
G	0.27	0.01	nd	0.01	0.17	0.01	0.03	0.01	0.50
I	0.14	0.05	0.03	0.02	0.07	0.07	0.04	0.02	0.28
J	0.07	0.01	nd	0.00	nq	0.01	0.03	0.01	0.14
K	0.07	0.01	nd	0.01	nq	0.00	0.03	0.01	0.14
L	0.06	0.01	nd	0.00	nd	0.00	0.03	0.00	0.12
M	0.20	0.20	nd	0.01	0.20	0.31	0.04	0.01	0.46
N	0.08	0.01	nd	0.01	nq	0.00	0.04	0.01	0.16
O	0.05	0.00	nd	0.00	nd	0.00	nd	0.00	0.08
P	0.06	0.00	nd	0.00	nq	0.00	0.03	0.00	0.11
Q	0.05	0.00	nd	0.00	nd	0.00	nd	0.00	0.09
R	0.06	0.01	nd	0.01	nq	0.00	0.03	0.00	0.12
S	0.06	0.04	dn	0.01	nq	0.05	0.03	0.02	0.14
T	0.05	0.02	nd	0.01	nq	0.01	0.03	0.02	0.11
AC	0.25	0.02	nd	0.01	0.08	0.01	0.09	0.01	0.50
AE	0.20	0.04	0.07	0.01	nq	0.01	0.07	0.01	0.40
AF	0.18	0.03	0.07	0.01	nq	0.01	0.06	0.01	0.36
AG	0.03	0.00	0.04	0.00	nd	0.00	nd	0.00	0.08
AH	0.06	0.03	0.08	0.03	nd	0.00	nd	0.01	0.17
AI	0.04	0.01	0.06	0.01	nq	0.00	nd	0.00	0.15
AJ	0.05	0.01	0.08	0.02	nq	0.01	nd	0.01	0.17
AK	0.05	0.01	0.07	0.01	nq	0.01	0.03	0.01	0.18
AL	0.03	0.00	nd	0.00	nd	0.00	nd	0.00	0.05
AM	0.04	0.01	nd	0.00	nq	0.00	0.05	0.01	0.12
AN	0.04	0.02	nd	0.01	nd	0.00	nd	0.01	0.08
AO	0.04	0.00	0.06	0.00	nq	0.00	nd	0.00	0.15
AP	0.07	0.02	0.10	0.02	nq	0.01	0.03	0.01	0.25
AQ	0.08	0.02	0.11	0.02	nq	0.01	0.05	0.01	0.28
AR	0.16	0.20	0.22	0.25	nq	0.04	0.04	0.03	0.47
AS	0.08	0.02	0.13	0.02	0.28	0.34	0.04	0.02	0.53
AU	0.06	0.01	0.05	0.03	nq	0.01	0.04	0.00	0.19
AV	0.05	0.01	0.06	0.04	nq	0.01	0.03	0.01	0.17
AW	0.07	0.02	0.03	0.02	nq	0.00	0.03	0.01	0.16
AX	0.05	0.01	nd	0.00	nd	0.00	0.03	0.02	0.11
BA	0.05	0.01	nd	0.00	nq	0.01	nd	0.01	0.10
BB	0.04	0.02	nd	0.01	nd	0.00	nd	0.00	0.09
BC	0.03	0.00	nd	0.00	nd	0.00	nd	0.00	0.07

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BD	0.05	0.00	nd	0.00	nq	0.01	0.03	0.01	0.13
BH	0.31	0.03	0.16	0.02	0.11	0.02	0.13	0.02	0.71
BI	0.27	0.05	0.13	0.02	0.09	0.02	0.11	0.02	0.59
BJ	0.41	0.29	0.22	0.16	0.15	0.10	0.17	0.11	0.96
CA	0.51	0.07	0.35	0.06	0.28	0.10	0.39	0.17	1.52
CB	0.51	0.09	0.34	0.06	0.21	0.04	0.27	0.05	1.33
CC	0.39	0.02	0.25	0.01	0.15	0.01	0.20	0.01	0.98
CD	0.48	0.06	0.30	0.03	0.19	0.02	0.23	0.05	1.20
CE	0.36	0.03	0.21	0.02	0.14	0.02	0.14	0.01	0.85
CF	0.53	0.08	0.32	0.05	0.21	0.05	0.22	0.04	1.27
CG	0.30	0.00	0.17	0.00	0.12	0.00	0.12	0.00	0.70

nd: Not detected

nq: Not quantifiable

Table 4.5: *The level of 4PAH in international wines (n=13) as well as the standard deviations (SD)*

Wine code	**Chry**		**B[*a*]A**		**B[*b*]F**		**B[*a*]P**		**∑4PAH**
	Conc (ppb)	**SD**	**Conc (ppb)**	**SD**	**Conc (ppb)**	**SD**	**Conc (ppb)**	**SD**	**Conc (ppb)**
U	0.15	nd	0.05	nd	0.04	nd	0.05	nd	0.29
V	0.18	nd	0.06	nd	0.05	nd	0.06	nd	0.36
W	0.11	0.03	0.03	nd	0.03	nd	0.04	nd	0.22
X	0.13	0.05	0.04	nd	0.04	nd	0.04	nd	0.26
Y	0.12	nd	0.03	nd	0.03	nd	0.04	nd	0.22
Z	0.13	nd	0.04	nd	0.03	nd	0.04	nd	0.24
AA	0.33	nd	0.16	nd	0.11	nd	0.13	nd	0.73
AB	0.23	0.04	0.10	nd	0.09	0.02	0.09	nd	0.51
AD	0.19	nd	0.07	nd	0.06	nd	0.06	nd	0.38
AY	0.04	nd	0.01	nd	0.00	nd	0.02	nd	0.07
AZ	0.05	nd	0.02	nd	0.01	nd	0.02	nd	0.10
BG	0.24	nd	0.13	nd	0.09	nd	0.10	nd	0.56
CH	0.38	nd	0.22	nd	0.15	nd	0.16	nd	0.91

nd: not detected

Of the 57 South African wines analysed, 11 of the wines had PAH levels between 0.50 and 1 ppb (for the sum of the 4 PAHs), and 6 of the wines had levels higher than 1ppm. Of the international wines, four wines had PAH levels between 0.50 and 1 ppb (for the sum of the 4 PAHs).

The South African wines with levels of 4PAH between 0.50 and 1 ppb came from Stellenbosch (7), Paarl (3) and the Swartland (1). Nine of the wines were red wine (7 blends, 1 Shiraz and 1 Merlot) and two wines were white wine (Sauvignon blanc and Chardonnay). The vintages ranged from 2008 to 2012. The majority of the wines were barrel aged, in a mixture of first, second and third fill barrels.

The wines that had levels of 4PAH above 1 ppb include 6 South African wines. Three of these were Shiraz wines from the same wine estate in Stellenbosch (vintage 2005, 2006, and 2007). Another of the wines was a Cabernet Sauvignon, 1980, from Stellenbosch. The other two wines

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came from Paarl and Wellington respectively. The wine from Paarl was a sweet red blend, 2010, and the wine from Wellington was a Shiraz, 2008.

The international wines with levels of 4PAH between 0,50 and 1 ppb included 4 wines, from Germany, Spain, Italy and Australia. Two wines were red varieties and 2 were white varieties, from 2010 and 2011.

Further patterns between the PAHs in the wines were examined using principal component analysis plots (4.3.2).

There were no significant differences between the South African wines and the international wines. Figure 4.2 shows the differences in the B[*a*]A, B[*b*]F, B[*a*]P and Chry for South African and international wines, showing no significant differences ($p \geq 0.05$) for all four PAHs.

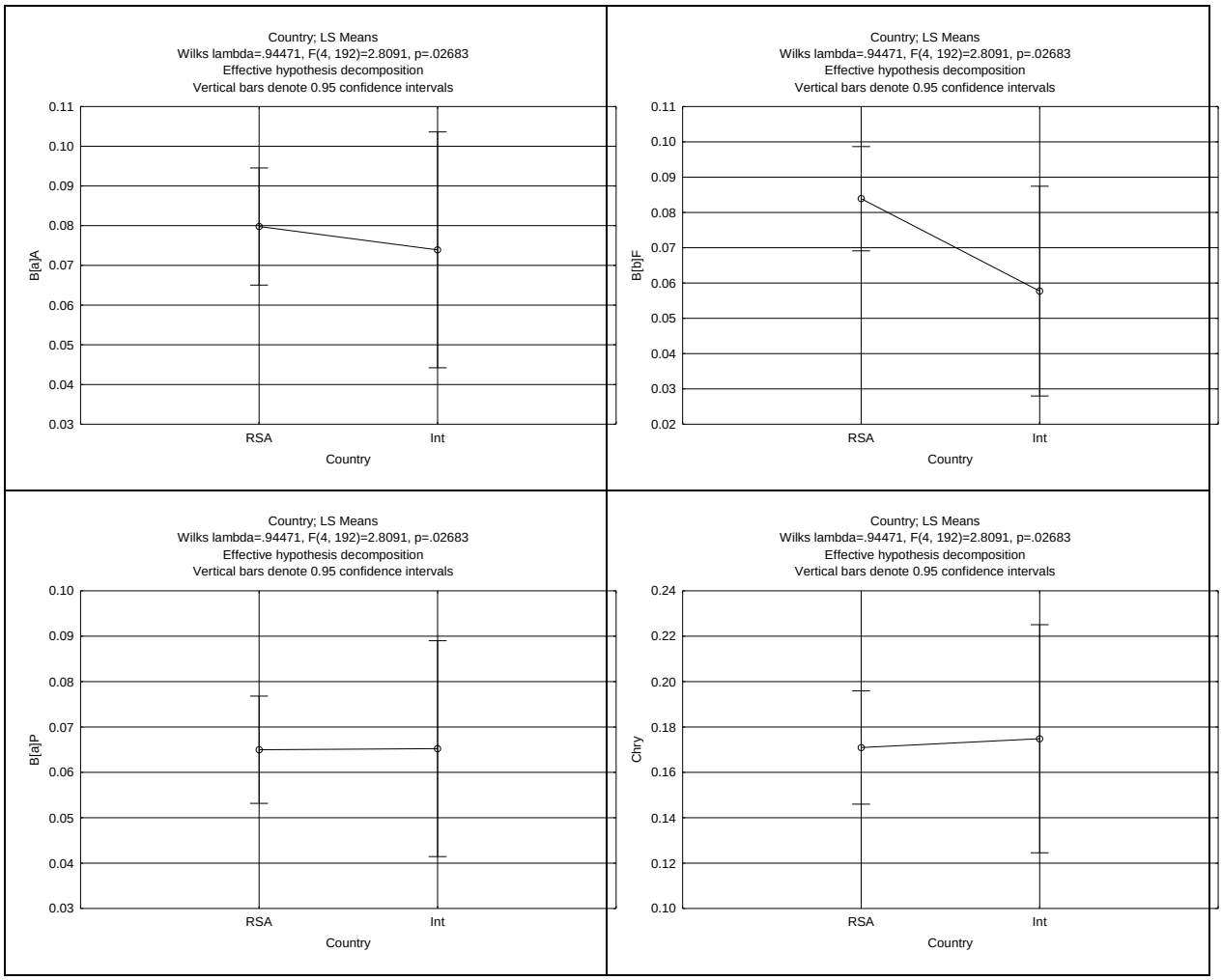

Figure 4.2: The differences between South African and international wines for B[*a*]A, B[*b*]F, B[*a*]P and Chry at a confidence interval of 95%.

4.3.2 Principal component analysis (PCA)

The South African wines were plotted in Figure 4.3 on a PCA graph. 80% of the variation in the sample set was explained by first principal component (PC1). Four wines, CA, CB, CD and CF were positively correlated to PC1 (the majority of the PAHs). Wines CC, CE and CH also correlate

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positively to PC1, although not as strongly as CA, CB, CD and CF. The second principal component (PC2) explained only 9% of the variation in the sample set, making little contribution to the model. This is explained by wine E's higher level of B[*b*]F.

Wines CA, CB and CC were Shiraz wines from the same wine estate. This estate, like other estates from where wine was sourced for analysis, used creosote posts. The only differences between wines CA, CB and CC were that they were from 2005, 2006 and 2007 vintages respectively. Wine CE was a 2009 Merlot from the same estate. The wines from this estate come from single vineyard blocks, and are aged in French oak barrels for 16 – 18 months. A variety of factors may therefore have potentially contribute to PAHs in the wines. The levels of PAHs detected in these wines was however still below the acceptable limit.

Wine CD was a sweet fortified Pinotage from Paarl (2010), with no exposure to creosote in the vineyards, and had been aged for 11 months in older barrels. Wine CF was a Shiraz 2008 from Wellington, made from grapes grown in the vicinity of creosote posts. The wine was aged in third fill barrels for 12 months. Bushfires were prevalent in the South African harvest of 2008, so wines from that vintage may have been exposed to environmental contamination from bushfires. Wine CH was an Australian Shiraz, 2008 from Margaret's River. This wine was not exposed to bushfires that season, nor exposed to creosote posts in the vineyard. The wine was aged for 18 months in French oak barrels, 30% of which were new.

Wine E has higher levels of B[*b*]F – this wine is a red blend (Shiraz/Cabernet Sauvignon/Merlot) 2007, from the Swartland region, where some of the grapes were exposed to creosote. The wine was aged for 20 months in French barrels. Again, these potential sources of PAH contamination have not resulted in levels of PAHs in the wine that exceed acceptable limits.

Of the 8 wines highlighted by the PCA graphs, five of the wines were Shiraz and one a Shiraz blend. The other two wines were Pinotage and Merlot.

Despite the cultivation, environmental and winemaking factors mentioned above, and that have been noted in literature to potentially contribute to PAH contamination in wine, none of the wines analysed in this study had levels of PAHs that posed a health risk. Most of the levels were below the legal limits set by the Czech Republic. All of the wines were below the limits set by the European Union of 1 ppb B[*a*]P for all dietary food (European Directive 2006/125/EC) and lower than the guidelines offered by AFFSA of 2.0 ppb.

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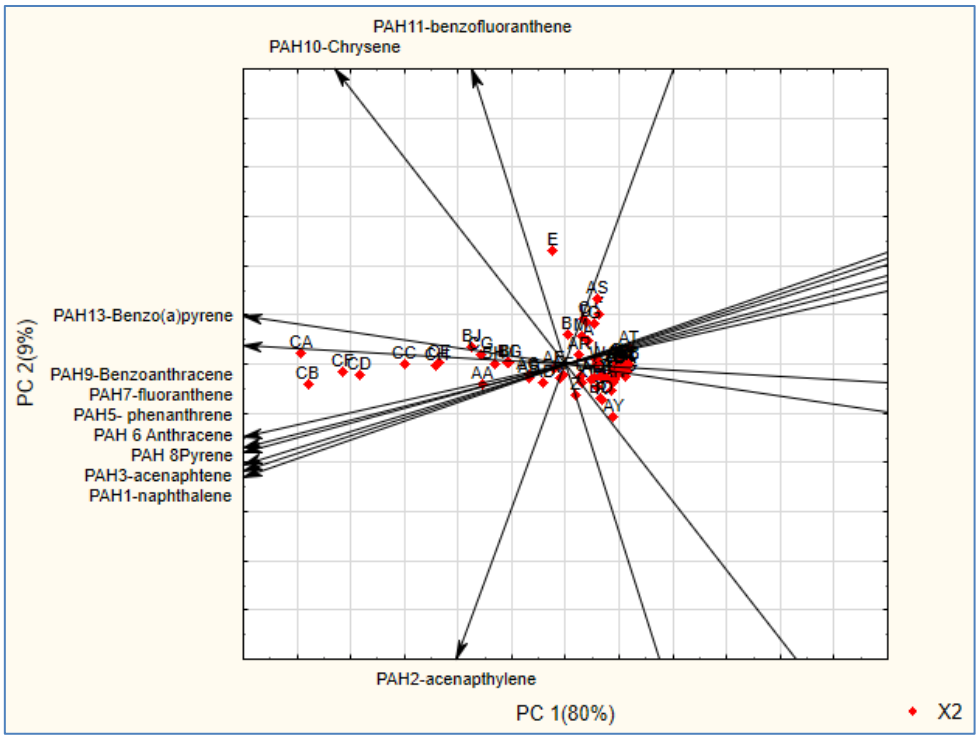

Figure 4.3: PCA graph examining patterns in PAH concentration.

Figure 4.4 compares the international wines to the South African wines. On average, there is little difference between the two sample sets (only within 9%), as the confidence ellipses overlap. The outliers are mostly the South African wines explained by the PCA graph in figure 4.3.

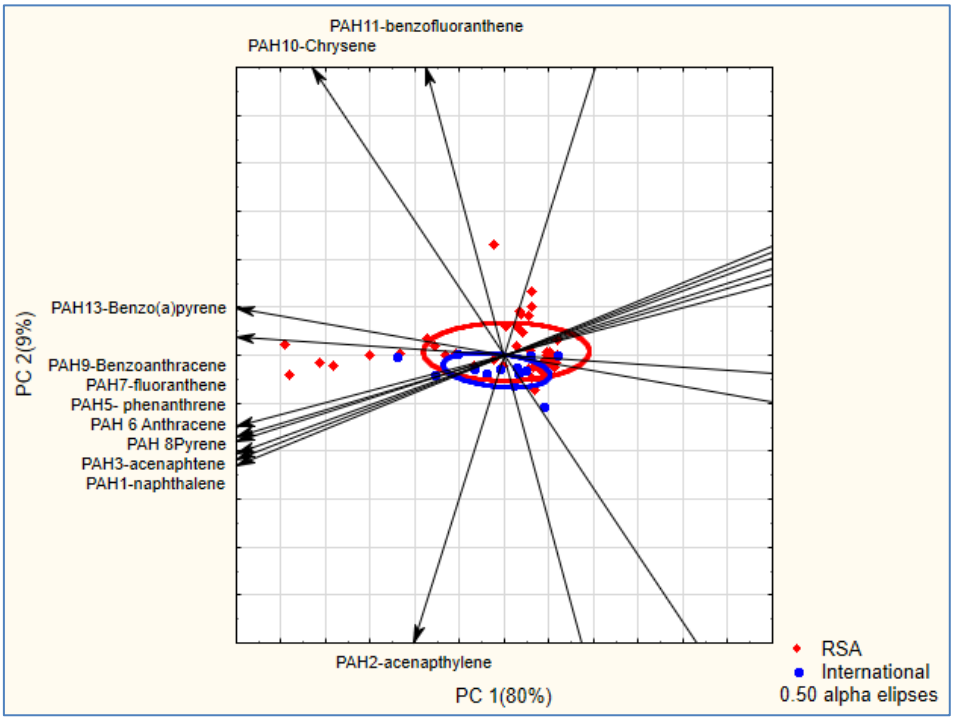

Figure 4.4: PCA graph comparing South African and international wines.

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4.4 Conclusions

The South African and international wines analysed have relatively low levels of 4PAHs.The quantities are in most cases less than the legal limit of 0.50 ppb in the Czech Republic and in all cases lower than the guidelines offered by AFFSA of 2.0 ppb, indicating that these wines do not pose a health risk. There are no significant differences between PAH4 levels in South African and international wines.

A few South African wines have higher levels of PAH than others. However, these levels fall below levels that warrant further investigation. The potential cause of these slightly elevated levels may be from exposure to creosote posts used in these vineyards. It may also be that the vineyards are in close proximity to busy roads (vehicle emissions) or industrial activity (atmospheric pollution), or were exposed to smoke from bush fires. There may be an accumulative effect: wines made from grapes grown in vineyards with creosote that are nearby roads with heavy traffic, together with exposure to smoke from bush fires, and in addition are aged in barrels, may lead to higher wine PAH levels. However, the PAH levels are low and fall within recommended guidelines offered by AFFSA.

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Chapter 5

General discussion and conclusions

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CHAPTER V: GENERAL DISCUSSION AND CONCLUSIONS

General discussion

PAHs are known for their strong mutagenic and carcinogenic properties and are potent immunosuppressants. They are organic pollutants, resistant to degradation. They occur in all parts of the environment, from the atmosphere, water, soil and vegetation, impacting the environment adversely, and affecting the health of people who come into contact with PAHs (EFSA, 2008). For these reasons, PAHs are monitored in food, water and air, with most European Union countries and the United States having strict regulations regarding the legal limits allowed in foodstuffs and water (Wenzl *et al*., 2006)

The literature study examined the general environmental sources of PAHs, which, according to the PAH Positions Paper 2001 (OOPEC, 2001) can be divided into 5 categories: domestic, mobile, industrial, agricultural and natural sources. The study focused on the sources which might expose wine to PAHs – either in viticulture or in the cellar. It was found that agricultural land may be exposed to PAHs through two main sources – contaminated water used for irrigation and atmospheric pollution (through vehicle and industry emissions as well as bush fires). It was also found that grapes could be exposed to PAHs through the use of creosote posts in the vineyards.

Grapes or wine could be exposed to PAH contamination in the cellar – through the use of toasted wine barrels and possibly through accidental oil leaks due to faulty equipment.

Chapter 3 compared the use of the Ultra high-pressure liquid chromatography (UPLC) and high-pressure liquid chromatography (HPLC) methods for determination polycyclic aromatic hydrocarbons (PAHs), using a liquid-liquid extraction method (LLE). Clean wines were spiked with a PAH mix of 12 priority PAHs, at five concentration levels. The PAHs were extracted from the wine and analysed on both instruments. Linearity, precision, limits of detection (LOD), limits of quantification (LOQ) and recovery were measured and compared. Both instruments showed a satisfactory linearity. The LOD and LOQ values, as well as the recovery fell within the recommended range. Precision of the instruments were tested by analysing pure calibration standards at 50 ppb, 6 times, and comparing the relative standard deviation of the recovery of PAHs. The RSDs for compounds were below 2.3%, indicating that the precision of the instruments is within an acceptable range.

Chapter 4 focused on the determination of the levels of PAHs in wine. The 12 priority PAHs were monitored, with a focus on the four PAHs that have been chosen as indicators for the occurrence of PAHs in food by the European Food Safety Agency, 4PAH: chrysene, benzo(*a*)anthracene, benzo(*b*)fluoranthene and benzo(*a*)pyrene (EFSA, 2008). There are no legal limits set for PAH levels in wine. The PAH limit for drinking water, set by the Commission Regulation 98/83/EC, is 0.01 ppb for B[*a*]P and 0.1 ppb for 4PAH. When compared to this, as well as the legal limits set

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for smoked and char-broiled foodstuffs (10 – 35ppb), the wines analysed have relatively low levels of 4PAH. The levels for 4PAH ranged from 0.05 to 1.52 ppb, and there were no significant differences between wines from South Africa and the international wines analysed.

Conclusions

It can be concluded that in general, the wines analysed in this study were safe for human consumption and complied with the EU regulations. Although the wines with the highest levels of B[*a*]P and B[*a*]A were mostly made from grapes grown in vineyards that still use creosote posts for trellising, other factors such as exposure to smoke from bushfires, vehicle emmisions and industrial pollution, as well as winemaking practices may also contribute to these PAH levels. Although creosote posts have been associated with PAH contamination in literature, this study shows that of the levels of PAHs in the wines was not above the recommended limit.

This exposure to PAH contamination may contribute to an increase in PAH levels in the final product. The Integrated Production of Wine (IPW) advises against the use of creosote posts for trellising in vineyards in South Africa. Together with the increase of PAHs in wine, creosoted posts still have a negative effect on the soil and water (Van Zyl, 2013), as they leach PAHs into the environment.

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