APPLICATIONS OF LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY TO WINE ANALYSIS: TARGETED ANALYSIS AND COMPOUND IDENTIFICATION

P. Alberts

Dissertation presented for the degree of

Doctor of Philosophy (Chemistry)

at

Stellenbosch University

Dr. A. J. de Villiers (supervisor) Stellenbosch

Dr. M. A. Stander (co-supervisor) December 2012

Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:_____

Date:_____

Summary

The wine industry is an important sector of agriculture and wine analysis forms the basis of assessing compliance of its commodities with regulatory standards and research in this field. Liquid chromatography (LC) is extensively used for the determination of a wide range of non-volatile wine components, but conventional detectors impose performance limitations on the technique that prevents its application to sophisticated analytical problems. In particular, conventional detectors for LC often lack the sensitivity and specificity for the determination of many wine compounds, especially trace level analytes, and furthermore, do not possess spectral capabilities for compound identification or structure elucidation. The hyphenation of mass spectrometry (MS) to LC has led to the introduction of a range of detectors that confers high levels of sensitivity and selectivity to the technique. In addition, a wide variety of MS architectures are available that are inherently suited for targeted analysis or structure elucidation studies.

In this dissertation, the potential benefits of liquid chromatography – tandem quadrupole mass spectrometry (LC-MS/MS) to solve analytical problems relevant to the wine industry are explored. LC-MS/MS is a particularly versatile analytical technique because both mass analysers can be operated in full-spectrum mode or selected-ion monitoring, which, together with optional fragmentation, gives rise to four modes of operation that may be used for highly specific and sensitive targeted analysis or spectral investigations.

In multiple reaction monitoring (MRM) mode, both analysers are set at single ion frequencies specific for the compound under investigation and one or more of its product fragments, respectively. MRM mode is ideally suited for trace level analysis in complex mixtures, even in cases where the target components are not resolved from interferences. In this study, MRM detection was used to solve challenges relevant to the wine industry for the selective quantitation of target analytes that could not be analysed by conventional LC methods. The application of this approach for the analysis of natamycin, ethyl carbamate (EC) and 3-alkyl-2-methoxypyrazines (MPs) in wine is demonstrated.

Natamycin is an antimicrobial preservative that is not permitted in wine in the European Union. A rapid and sensitive method for the determination of natamycin was developed, and has been

used since 2009 to regulate this vitally important sector of the South African wine export industry.

EC is a natural carcinogen that occurs at trace level amounts in alcoholic products. It also has the potential to accumulate in wines and can occur in very high concentrations in some fruit brandies. The determination of EC is complicated by its physicochemical properties, and available analytical methods suffer from drawbacks such as the requirement for elaborate extraction procedures and high solvent consumption. A novel method for the determination of EC in wines, fortified wines and spirits is described and it was applied to perform an audit of the South African industry as well as to investigate factors responsible for its accumulation in alcoholic beverages. This work forms an integral part of the food safety mandate of the State and it ensures that export products comply with international norms for trade.

MPs are ultra-trace-level aroma compounds that contribute to the varietal character of Sauvignon blanc wines. Their analytical determination is challenging due to their low levels of occurrence. The loading capacity of LC combined with the sensitivity and resolving power of MS was exploited to analyse concentrated extracts, in order to achieve very low limits of detection. The performance of the LC-MS/MS method enabled the quantitation of these compounds at their natural levels of occurrence, including the first quantitation and spectral confirmation of 3-ethyl-2-methoxypyrazine in wine. Extensive data pertaining to South African Sauvignon blanc wines are reported and statistical analysis is performed, reporting the correlation of variables such as vintage and origin as well as wine parameters such as malic acid with wine MPs.

Furthermore, the application of LC-MS/MS for structural elucidation and screening of target classes of analytes was demonstrated for the analysis of red wine anthocyanins. The anthocyanidin-glycosides are responsible for the colour of red grapes and wine, contribute to the sensory properties of wine, and are also of interest due to their beneficial biological properties. Their determination is complicated by their large numbers and structural diversity, further exacerbated by diverse reactions during wine ageing as well as the lack of reference standards for most members of this class of compounds. Tandem MS in scan mode was used for the highly selective detection of glycosylated anthocyanins and derivatives, exploiting the predictable elimination of the sugar moiety in neutral loss mode. Concurrent survey scan experiments were used to unambiguously identify neutral loss detected compounds. The method therefore follows a simplified and structured approach for unambiguous peak

identification based on elution order and mass spectral information to impart a high level of certainty in compound identification.

In summary, the work presented in this dissertation demonstrates that LC-MS/MS is a versatile and powerful analytical approach for the analysis of diverse compounds of relevance to the wine industry. The sensitivity and specificity of MRM mode, and the selectivity and spectral capabilities of neutral loss and survey scan modes of MS/MS detection, is amply demonstrated by the applications presented in the dissertation.

Opsomming

Die wynbedryf is ‘n belangrike komponent van landbou en wyn-analise vorm ‘n integrale deel van gehalteversekering ten opsigte van toepaslike wetlike standaarde. Wyn-analise is ook belangrik in navorsing oor die samestelling van wyn. Vloeistofchromatografie word dikwels aangewend vir die bepaling van ‘n wye verskeidenheid nie-vlugtige wynkomponente, maar konvensionele detektors plaas beperkinge op die aanwending van die tegniek tot gesofistikeerde analitiese toepassings. Meer spesifiek, konvensionele detektors vir vloeistofchromatografie beskik nie oor die sensitiwiteit en selektiwiteit vir die bepaling van baie wynkomponente nie, veral in die geval van spoorvlakanalise, en beskik boonop ook nie oor spektrale vermoëns vir identifikasie van komponente en struktuurbepaling nie. Die koppeling van vloeistofchromatografie met massaspektrometrie het ‘n reeks detektors tot die tegniek toegevoeg wat hoë vlakke van sensitiwiteit en selektiwiteit bied. Verder bied die verskeidenheid van massaspektrometrie-konfigurasies ook instrumente wat inherent geskik is vir geteikende analise of struktuurbepaling, afhangende van die doel van die ondersoek.

In hierdie dissertasie word die voordele ondersoek wat verbonde is aan die aanwending van vloeistofchromatografie – tandem kwadrupool massaspektrometrie om relevante analitiese vraagstukke in die wynbedryf op te los. Hiedie tegniek is besonder toepaslik aangesien beide massa-analiseerders in geselekteerde-ioon modus of in volle skandering gebruik kan word. Tesame met opsionele fragmentasie, gee hierdie uitleg aanleiding tot vier funksionaliteite wat vir hoogs sensitiewe geteikende analise of spektrale onledings gebruik kan word.

Eerstens word beide massa analiseerders vir enkel-ioon frekwensies opgestel, spesifiek tot die teikenkomponent en een of meer van sy produkfragmente, wat verkry word deur komponent-spesifieke fragmentasie. Hierdie modus is by uitstek geskik vir spoorvlakontleding van komplekse monsters, selfs wanneer die teikenkomponente nie chromatografies van die matriks geskei is nie. In hierdie studie is die tegniek aangewend vir die hoogs sensitiewe bepaling van spoorvlak komponente wat nie met konvensionele detektors gemeet kon word nie. Die aanwending van hierdie tegniek word gedemonstreer vir die spoorvlakbepaling van natamycin, etielkarbamaat en 3-alkiel-2-metoksiepierasiene in wyn.

Natamycin is ‘n antimikrobiese preserveermiddel wat ontoelaatbaar is in wyn in die Europese Unie. ‘n Vinnige en sensitiewe metode vir die bepaling van natamycin is ontwikkel, en word

reeds sedert 2009 aangewend om hierdie uiters belangrike sektor van die Suid-Afrikaanse wyn uitvoerbedryf te reguleer.

Etielkarbamaat is ‘n karsinogeen wat natuurlik voorkom in spoorhoeveelhede in alkoholiese produkte. Dit kan ook onder sekere omstandighede akkumuleer in wyn en in hoë konsentrasies voorkom in vrugtebrandewyne. Die bepaling van etielkarbamaat word bemoeilik deur sy chemiese eienskappe, en gevolglik word analitiese metodes gekenmerk deur uitgebreide, arbeidsintensiewe monstervoorbereiding en die gebruik van groot hoeveelhede, meestal giftige, oplosmiddels. ‘n Nuwe metode vir die bepaling van etielkarbamaat in wyn, gefortifiseerde wyn en spiritualië word beskryf en word aangewend om die faktore vir vorming daarvan te ondersoek. Die metode word aangewend om die Suid-Afrikaanse bedryf te ouditeer in terme van die voedselveiligheid mandaat van die Staat, en om te verseker dat uitvoere voldoen aan standaarde vir internasionale handel.

Metoksiepierasiene is vlugtige, ultraspoorvlak wynaromakomponente wat verantwoordelik is vir die kenmerkede kultivarkarakter van Sauvignon blanc wyne. Hul analitiese bepaling word bemoeilik deur hulle lae konsentrasies in wyn. Die ladingskapasiteit van vloeistofchromatografie tesame met die sensitiwiteit en selektiwiteit van massaspektrometrie was benut om hoogs gekonsentreerde ekstrakte te ontleed. Baie hoë vlakke van sensitiwiteit word sodoende verkry. Die verrigting van die metode was voldoende om hierdie komponente teen hulle natuurlike konsentrasies te kwantifiseer, insluitende die eerste kwantifisering en spektrale bevestiging van 3-etiel-2-metoksiepierasien. Omvattende data van die vlakke van hierdie komponente in Suid-Afrikaanse Sauvignon blanc wyne word getoon en statistiese ontleding is gedoen om korrelasies tussen veranderlikes soos oorsprong en oesjaar sowel as basiese wyn veranderlikes soos byvoorbeeld appelsuur, met metoksiepierasienvlakke te ondersoek.

Verder was die toepassing van vloeistofchromatografie – tandem massaspektrometrie tot struktuurbepaling en skandering vir groepe van komponente gedemonstreer vir die ontleding van rooiwyn antosianiene. Die antosianien-glukosiede is verantwoordelik vir die kleur van rooi druiwe en wyn, dra by tot die sensoriese eienskappe daarvan, en is ook relevant as gevolg van die voordelige biologiese eienskappe daarvan. Die bepaling van hierdie komponente word gekompliseer deur hulle groot getalle en strukturele diversiteit, verder bemoeilik deur die wye verskeidenheid van reaksies wat hulle ondergaan tydens veroudering. Daar is ook ‘n gebrek aan beskikbaarheid van standaarde vir die meeste van die lede van hierdie klas van

komponente. Tandem massaspektrometrie was in skanderingsmodus gebruik vir hoogs selektiewe deteksie van die antosianien-glukosiede deur die voorspelbare eliminasie van die suiker komponent in neutrale verliesskandering te benut. Gelyktydige skanderings van die komponente wat met neutraleverliesskandering waargeneem word, is gebruik vir ondubbelsinnige komponent identifikasie. Die metode volg daarom ‘n eenvoudige en gestruktureerde benadering vir piek identifikasie wat gebaseer is op chromatografiese orde, sowel as massaspektrale inligting, om ‘n hoë vlak van sekerheid aan die identifikasie van komponente te verleen.

Samevattend, word daar getoon deur die werk wat in hierdie dissertasie uiteengesit is dat vloeistofchromatografie – tandem massaspektrometrie ‘n veelsydige en kragtige tegniek bied vir chemiese analise relevant tot die wynbedryf. Die sensitiwiteit, selektiwiteit en spektrale vermoëns van die tegniek word duidelik deur toepassings in die dissertasie getoon.

Acknowledgements

I would like to express my gratitude to the following persons and institutions:

- The supervisors for the project, Drs. A.J. de Villiers and M.A. Stander.
- The Department of Agriculture, Forestry and Fisheries, in particular Mr. A. Smith for facilitating the project.
- The Wine and Spirit Board of South Africa for providing samples of Sauvignon blanc wine.
- For their kind assistance with method development and sample preparation, my colleagues A. le Roux, L. Soboyisi, J. Waries and T. Swart.

Abbreviations

AAS	Atomic absorption spectroscopy
ABTS	2,2'-Azinobis(3-ethylbenzothialozinesulfonic acid)
Alc	Alcohol content
Amu	Atomic mass units
ANOVA	Analysis of variance
AOTF	Acousto-optical tunable filter instrument
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photo-ionisation
ATR	Attenuated total reflection
BGE	Background electrolyte
BWI	Biodiversity and Wine Initiative
CAR	Carboxen
ca.	Circa
CE	Capillary electrophoresis
cGC	Capillary gas chromatography
CI	Chemical ionisation
CID	Collision induced dissociation
CL	Confidence limits
Cy	Cyanidin
DAD	Diode array detector
DC	Direct current
DCM	Dichloromethane
Dp	Delphinidin
DPPH	2,2-Diphenyl-1-picrylhydrazyl radicals
DVB	Divinylbenzene
EC	Ethyl carbamate
ECD	Electron capture detector
EI	Electron impact ionisation
ELSD	Evaporative light scattering detector
EMP	3-Ethyl-2-methoxypyrazine
ESI	Electrospray ionisation
EU	European Union
FA	Factor Analysis
FFAP	Free fatty acid phase
FID	Flame ionisation detector
FL	Fluorescence detection
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
FTIR	Fourier transform infrared spectroscopy
FLD	Fluorescence detector
FT-MIR	Fourier transform mid-infrared spectroscopy
FT-NIR	Fourier transform near-infrared spectroscopy
FWHM	Full width at half maximum height
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometry
GC-O	Gas chromatography – olfactometry
GDP	Gross domestic product
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
HSSE	Headspace sorptive extraction
HS-SPME	Headspace solid phase micro-extraction
HTLC	High temperature liquid chromatography
IBMP	3-Isobutyl-2-methoxypyrazine

ICP-MS	Inductively coupled plasma – mass spectrometry
ID	Internal diameter
IPMP	3-Isopropyl-2-methoxypyrazine
IPW	Integrated production of wine
IR	Infrared
KWV	*Koöperatieve Wijnbouwers Vereniging van Zuid-Afrika Bpkt*
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LDA	Linear discriminant analysis
LIT	Linear two-dimensional ion trap
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI	Matrix assisted laser desorption ionisation
MIR	Mid-infrared
MMP	3-Methyl-2-methoxypyrazine
MP	3-Alkyl-2-methoxypyrazine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
Mv	Malvidin
m/v	Mass per volume
MW	Molecular weight
m/z	Mass to charge ratio
NIR	Near-infrared
NMR	Nuclear magnetic resonance
NPD	Nitrogen-phosphorus detector
OIV	*Office International de la Vigne et du Vin*
OTTs	Open tubular traps
PC	Principal component
PCA	Principal component analysis
PCR	Principal component regression
PDMS	Polydimethylsiloxsane
Pe	Peonidin
PEG	Polyethyleneglycol
PFPD	Pulsed flame photometric detector
PLS	Partial least squares regression
PSDVB	Polystyrene-divinylbenzene
Pt	Petunidin
Q	Quadrupole analyser
QTOF	Quadrupole time-of-flight
QuEChERS	Quick, easy, cheap, effective, rugged and safe method
REA-PFGE	Endonuclease analysis pulsed field gel electrophoresis
RF	Radio frequency
RI	Refraction index
RMSEP	Root mean square error of prediction
RP	Reversed phase
RPD	Residual predictive deviation
RP-LC	Reversed phase liquid chromatography
RSD	Relative standard deviation
SAWIS	South African Wine Industry Iinformation and Systems
SBMP	3-*sec*-Butyl-2-methoxypyrazine
SBSE	Stir bar sorptive extraction
SEP	Standard error of prediction
SDB	Styrene-divinylbenzene
SIM	Selected ion monitoring

SIMCA	Soft independent modelling of class analogy
S/N	Signal-to-noise ratio
SPDE	Solid phase dynamic extraction
SPE	Solid phase extraction
SPME	Solid phase micro-extraction
TA	Titratable acidity
TIC	Total ion chromatogram
TOF	Time-of-flight
TSS	Total soluble solids
UHPLC	Ultra high pressure liquid chromatography
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
UV/Vis	Ultraviolet/visible
VA	Volatile acidity
v/v	Volume per volume
WHO	World Health Organisation
WO	Wine of Origin scheme

Note

This dissertation is presented as a compilation of manuscripts already published or submitted for publication. Each manuscript is a chapter of an individual entity and some repetition between chapters has therefore been unavoidable.

List of publications:

1. A. de Villiers, P. Alberts, A.G.J. Tredoux, H.H. Nieuwoudt, Anal. Chim. Acta 730 (2012) 2-23 (Chapter 4).
2. P. Alberts, M.A. Stander, A. de Villiers, S.A. J. Enol. Vitic. 32 (2011) 51-59 (Chapter 5).
3. P. Alberts, M.A. Stander, A. de Villiers, J. Food Add. Contam. A 28 (2011) 826-839 (Chapter 6)
4. P. Alberts, M. Kidd, M.A. Stander, H.H. Nieuwoudt, A.G.J. Tredoux, A. de Villiers, (2012) submitted to S. Afr. J. Enol. Vitic. (Chapter 7).
5. P. Alberts, M.A. Stander, A. de Villiers, J. Chromatogr. A 1235 (2012) 92-102 (Chapter 8).

Table of contents

Chapter 1

Introduction and objectives

1.1. Historical overview of the South African wine industry

Viticulture was introduced into South Africa in the 17th century by the Dutch when Jan van Riebeeck was sent to the Cape of Good Hope to establish a supply station for the Dutch East India Company, serving ships on the sea passage between Europe and the Indies. The purpose of the supply station was to provision ships operating on the spice route with fresh commodities to reduce the risk of scurvy. Vines were imported from Europe and the first harvest and crushing took place in 1659, seven years after his landing in 1652. The arrival in ca. 1688 of 200 French Protestant Huguenot refugees injected vital wine-making expertise into the emerging industry. In the late 18th and early 19th centuries the Cape wine industry became famous for Constantia, a sweet, fortified wine that achieved great acclaim in Europe.

Starting in 1861, the South African wine industry went into a decline when Britain removed import controls, making her market accessible to French products, and as a result of the Phylloxera epidemic (1866), which destroyed many of the Cape vineyards. By 1900 the industry had recovered to such an extent that it overproduced massive volumes of wine for which no market existed. Stability was restored with the formation of the *Koöperatieve Wijnbouwers Vereniging van Zuid-Afrika* (KWV), which was empowered to limit production and set minimum prices – developments that favoured increased production of brandy and fortified wines. By the mid 1980s these restrictions were eased to permit importation of improved vine cuttings, thereby introducing trends such as the production of Bordeaux-style blends to the industry.

South Africa made an important contribution to the history of the *vinifera* vine when Professor Perold of Stellenbosch University successfully crossed Pinot noir and Hermitage (the latter currently recognised as Cinsaut) in 1925 to create Pinotage, a uniquely South African cultivar. The transformation of the industry was also advanced by the development of local scientific and technological expertise such as cold fermentation processes (1957), which improved the quality of especially white wines.

In modern times the South African wine industry has continued to develop and since the transition to democracy, wine exports have proliferated, mainly to the United Kingdom and Europe. Wine exports from South Africa over this period increased from 855 000 cases in 1990 to 15.4 million cases in 2000 [1,2]. Currently 101 016 hectares of vines producing wine grapes are under cultivation in South Africa [3].

1.2. Economic importance of the South African wine industry

In terms of global fresh fruit production, grapes are the most important commodity, with approximately 70% of the yield being fermented into wine. Europe, particularly France, Italy and Spain, is the worlds' largest producer of wine [1]. South Africa is currently the 9th largest wine producing country in the world and 3rd largest in the southern hemisphere. In 2009, South Africa produced 806 million liters of wine, or 2.9% of worldwide production. Exports, mostly to Europe, accounted for 49.1% of the wine produced in 2009 [3,4]. The commercial value of this commodity is demonstrated by the fact that almost half of the total production of Cape wines is exported. A study commissioned by the South African Wine Industry Information and Systems (SAWIS) showed that some 275 600 people were employed, both directly and indirectly, in the South African wine industry in 2008. The study also concluded that the wine industry contributes R 26.2 billion to the gross domestic product (GDP), while the growth in GDP contribution has consistently been no less than 10% per annum since 2003 [3]. Clearly, the wine industry is an important sector of the South African agricultural industry, and it is of critical importance to the economy of the Western Cape region in particular.

1.3. Regulation of the wine industry

Two predominant factors of critical importance to wine character and quality are origin (soil and climate) and viticulture. Of these, origin is considered to be of greater importance and therefore European wine-producing countries have long-standing systems for control of origin to protect both producers and consumers. Wine of Origin (WO) legislation was first introduced in the South African industry in 1973 and currently its administration is overseen by the Wine and Spirit Board, a government-appointed organisation tasked with regulating the industry. The South African WO system is based on, and compliant with, European standards, since that market is of vital importance to the local wine export industry. Certified wines are provided with a uniquely numbered seal which guarantees the accuracy of all information on the label. The composition and appearance of the label is also subject to regulations. In the South African system, certified wines and uncertified wines may be exported. However, all export wines are subjected to sensorial and chemical analysis. In the case of bottled wines, the concession is valid for a period of 12 months, while bulk wines are subjected to sensorial and chemical analysis on a per-consignment basis and this concession is valid for 42 days. The certification process involves vineyard inspections, cellar inspections (including extensive documentation of the entire vinicultural process), chemical analysis of basic wine parameters (to satisfy legal requirements) and tasting to ensure a minimum quality standard and varietal character.

In terms of relevant legislation (Liquor Products Act, Act 60 of 1989), wine may be certified for origin (region, district or ward, as appropriate), estate, vintage and grape variety (cultivar). Demarcation of origin is done with due consideration of soil, climate and ecological factors since these have a clear influence on the product characteristics. The names and borders of all authorised origins are defined by law and are officially published in the Government Gazette (Republic of South Africa). Wines certified for a specific origin must be produced entirely from grapes produced within that geographical delimitation. When a product is certified as an estate wine, all the wine must originate from and be fermented at a registered, demarcated estate. Wine may, however, be barrel matured and bottled at different establishments without losing its estate status, contrary to the French system. In addition, a registered estate may not vinify more than half of its production as non-estate grapes, while that part of the harvest that is designated as non-estate shall be separately demarcated in bulk and must be bottled under a non-estate label [2,3].

All of the approximately 75 approved cultivars used in South Africa belong to the species *Vitis vinifera*. Each cultivar possesses characteristics regarding adaptability to soil, climate and wine style, and therefore a close relationship often exists between origin and cultivar. Blended wines may be certified as varietal wine provided that the variety constitutes at least 85% of the blend and that at least 85% of the product comes from one harvest, with the balance coming from the preceding or subsequent years. Blends that do not claim single varietal status may state the varietal composition, while the actual percentage must be stated if one component of the blend represents <20% of the volume of the product. Since important changes occur in wine with ageing, vintage may serve as a guide to certain aspects of its character. Products certified for vintage must constitute at least 85% wine from that vintage (with the balance coming from the preceding or subsequent years as above). Non-certified wines may not use any vintage descriptions on the labels.

South Africa meets *Organisation Internationale de la Vigne et du Vin* (OIV) requirements on prohibition of additives and wine labelling. In South Africa, traditional-method sparkling wine is not labelled as Champagne but as *Méthode Cap Classique*; nor is Flor yeast fortified wine matured in a Solera system labelled as Sherry. The same principle also applies to Port wines. Contrary to most European systems, the South African WO system places no limitations on crop yields, fertiliser quantities or levels of irrigation. Chaptalisation (addition of sugar) and all other forms of enrichment are banned, but acidification is permitted [2,3].

1.4. The chemical composition of wine

Wine is a very complex mixture containing well in excess of 1000 identified compounds, including more than 160 different esters. Although much remains to be discovered, the principal chemical constituents that impart the distinctive character to wines are mostly known. The relationships between these compounds and the sensory properties of wine are more difficult to discover since sensory analysis is inherently subjective, and taste and aroma compounds, may interact in complicated ways to influence sensory perception. For example, a particular varietal aroma may only rarely be ascribed to one or a few volatile compounds and distinctive fragrances usually arise from the combined effect of many aromatic compounds. The majority of wine compounds are metabolic by-products of yeast activity during fermentation. However, grape-derived aromatic compounds often constitute those compounds that make one wine distinct from another [1].

While the basic flavour of wine depends on approximately 20 compounds, the subtle differences that distinguish one varietal wine from the next depend on the combined effects of a large number of compounds [1]. Wine contains approximately 0.8-1.2 g/L total aroma compounds, the most abundant of these being fusel alcohols, volatile acids and fatty acid esters. Despite being present in much lower concentrations, carbonyl compounds, phenols, lactones, terpenes, acetals, some hydrocarbons as well as sulphur and nitrogen compounds contribute more significantly to the unique sensory properties of wine. Most of these individual wine aroma compounds, at their natural levels of occurrence, play no role in the sensory characteristics of wine. However, in combination they may have a profound effect on wine aroma and are indeed responsible for unique differences in wine aromas.

Wine taste and mouth-feel are primarily due to a few compounds that occur at concentrations above 0.1 g/L such as water, ethanol, non-volatile acids (primarily tartaric, malic and lactic acids), sugars (mostly glucose and fructose) and glycerol. Tannins are important sapid substances in red wines, but occur in white wines in significant amounts only following maturation in oak cooperage. The colour of red wines may be attributed to anthocyanins, a complex group of plant pigments belonging to the flavonoid family. In general, the phenolic compounds undergo complex changes during maturation, imparting important characteristics to wines, including appearance, taste, mouth-feel and fragrance [1].

1.5. Chemical analysis in the wine industry

The wine industry is possibly subject to more regulations than most because of the great diversity and complexity of its products. In international trade, laws are passed to regulate the quality, authenticity, and health and safety of commodities. The most well known wine regulations are those pertaining to the geographical origin, vintage and cultivar of the product, and compliance in this regard is principally (but not exclusively) enforced by bureaucratic means. Regulated quality, and health and safety parameters are generally enforced by laws that involve the chemical composition of wines. Consequently, chemical analysis is the basis for ensuring conformity to these regulations. Analytical techniques used in the wine industry range from classical wet chemistry methods for the determination of parameters such as alcohol content, reducing sugars, volatile acidity and sulphur dioxide, to highly advanced instrumental methods.

Wine presents a highly complex sample matrix and chromatographic techniques, which are inherently suited for the separation of complex mixtures and quantitation of their components, are frequently used in wine analysis. Gas chromatography (GC) is principally used in the analysis and research of the volatile fraction of wines. High performance liquid chromatography (HPLC) has found widespread application in wine analysis due to the versatility and scope of the technique, and it is primarily applied to the analysis of non-volatile wine components. Fundamental research in this field increasingly requires analytical techniques that are capable of higher sensitivity and selectivity. As a consequence conventional chromatographic detectors such as the flame ionisation detector in GC and the ultraviolet-visible spectroscopic detector in liquid chromatography (LC), increasingly fall short of experimental requirements.

The hyphenation of mass spectrometry (MS) to chromatography has created a powerful set of tools that combines the scope and utility of chromatography with the sensitivity and specificity of MS, and which has higher resolving power than MS alone. The technique has also found widespread applicability in wine analysis as it offers increased sensitivity and selectivity compared to conventional detectors. Tandem mass spectrometry (MS/MS) in particular confers considerable versatility to liquid chromatography – mass spectrometry (LC-MS) since both mass analysers can be operated in scan mode or selected ion monitoring, which together with optional fragmentation, makes the technique suited for highly sensitive and selective targeted analysis, or compound identification and structure elucidation, depending on the goal of the investigation.

1.6. Objectives

In view of the importance of chemical analysis to the wine industry, and its requirements in terms of improved analytical techniques, the principal objective of this thesis was a detailed evaluation of the potential of liquid chromatography – tandem quadrupole mass spectrometry (LC-MS/MS) to solve relevant analytical challenges in the local wine industry. For this purpose, two distinct types of analysis were investigated in the context of wine analysis.

Firstly, LC-MS/MS was used for highly sensitive and selective targeted analysis. The goal of this work was to develop suitable methods for the analysis of natamycin, ethyl carbamate and methoxypyrazines in South African wine – each of which represent important challenges in this industry.

Secondly, the applicability of LC-MS/MS in various operational modes was investigated for structure elucidation of complex wine constituents. The goal of this work was to develop improved methods for the detailed analysis of the complex red wine anthocyanins.

References

[1] R.S. Jackson, Wine science: Principles, practice and perception, Academic Press, San Diego, U.S.A. (2000).
[2] J. Robinson, The Oxford companion to wine, Oxford University press, Oxford, U.K. (1999).
[3] Wines of South Africa (WOSA), Stellenbosch, South Africa.
[4] N. Uren, Wêreld en plaaslike nuus, South African wine industry and systems (SAWIS), Paarl, South Africa (2010).

CHAPTER 2

Liquid chromatography – mass spectrometry: Theory and instrumentation

2.1. Introduction

High performance liquid chromatography (HPLC) (and the technologically more advanced form, ultra high pressure liquid chromatography, UHPLC) is the most widely used of all analytical separation techniques and is compatible with most compounds that can be dissolved in a liquid [1,2]. This technique is inherently suited to yield information regarding the quantity (based on peak area or height) and complexity (number of peaks) of components in a mixture. However, identification is inconclusive when non-spectroscopic detection techniques are used, i.e., when identification is based only on retention time. The reverse situation applies to spectroscopic techniques, which principally yield qualitative information. Spectroscopic methods require relatively pure samples and it is often difficult to extract quantitative information. Mass spectrometry (MS) offers increased sensitivity and specificity compared to most analytical techniques and lends itself to the use of stable isotopes in analytical investigations [3]. The hyphenation of chromatographic and spectroscopic techniques therefore provides complementary information about the identities and concentrations of compounds in a mixture [3,4]. In particular, the hyphenation of MS to liquid chromatography (LC) creates a very powerful, rugged and versatile analytical tool as it combines the scope and utility of LC with the sensitivity and specificity inherent to MS [1,5,6]. In this chapter, a brief overview of the theoretical aspects of liquid chromatography – mass spectrometry (LC-MS) relevant to the results reported in this thesis is presented.

2.2. Analytical liquid chromatography

LC is a physical separation technique in which the solutes are selectively distributed between two immiscible phases, namely a liquid mobile phase flowing through a stationary phase bed. The chromatographic process occurs as a result of repeated sorption/desorption steps during the movement of the solutes along the stationary phase. Separation is then the result of different mobilities of the solutes as a consequence of differences in their distribution coefficients between these two phases [4]. In modern analytical LC (HPLC, UHPLC), the high mobile phase viscosity and low analyte diffusion practically limit the technique to the use of relatively short packed columns. However, compared to gases, liquids offer a far greater variety in terms of solvating capabilities and therefore greater scope for selectivity optimisation. Gases, in contrast, have more favourable kinetic properties and yield higher efficiencies in open tubular columns, such as used in capillary gas chromatography (cGC). As a consequence, LC separations are mostly performed at moderate efficiencies, with the column length limited by pressure considerations, but with high potential for selectivity optimisation derived from the appropriate selection of separation mode, stationary phase chemistry and mobile phase composition [4,7].

2.3. Migration rates of solutes in liquid chromatography

The mobility of the solutes is described by the equilibrium constants for the interactions by which they distribute themselves between the mobile and stationary phases. Ideally, the distribution constant (K) is constant over a wide range of concentrations, which results in characteristics such as symmetric Gaussian peak shapes and retention times that are independent of concentration [2]. The retention time (t_R) represents the total time that a solute spends in the column. The retention factor (k) is defined as the time that the solute spends in the stationary phase relative to the time it spends in the mobile phase [2,4]. The degree to which two solutes are separated is referred to as chromatographic resolution (R_s). Resolution is mainly determined by two factors: selectivity (α) and efficiency (N). Selectivity describes the physicochemical interactions between the stationary phase and the solutes, and has the greatest effect on resolution [8]. The selectivity factor (α) of a separation for two species A and B is defined as follows:

$$a = \frac{K_B}{K_A} = \frac{((t_R)_B - t_M)}{((t_R)_A - t_M)} \qquad (2.1)$$

where K_B and K_A are the distribution constants for the strongly and less strongly retained species, and t_R and t_M the retention times of the solute and an unretained peak, respectively [2]. Efficiency is dependent on the characteristics of the column such as length, particle size and uniformity of the stationary phase, and is measured in terms of the number of theoretical plates (N) or plate height (H). The resolution equation may also be written in terms of α, k and N as follows:

$$R_S = \frac{\sqrt{N}}{4} \cdot \frac{(a - 1)}{a} \cdot \frac{k_2}{1 + k_2} \qquad (2.2)$$

where k_2 the retention factor of the last eluting solute [2,8].

2.4. Column efficiency in liquid chromatography

Chromatographic separation is generally accompanied by dilution of the solute, a phenomenon commonly referred to as peak broadening. Peak broadening predominantly occurs in the column, but may also occur outside the column. The ultimate peak-width, as measured at the detector, is the result of all individual dispersion processes taking place in the chromatographic system, including the injector, connection tubing, column and detector. However, on-column peak broadening is the primary source of peak broadening in most optimised chromatographic systems [2,4,7]. The discussion that follows pertains specifically to on-column peak broadening and its effect on the measured efficiency.

Chromatographic peaks generally resemble Gaussian curves because variable residence times of the solute in the mobile phase leads to irregular migration rates, with a symmetric spread of velocities around the mean value. The extent of peak broadening determines the chromatographic efficiency. The width of a Gaussian curve is directly related to the variance of measurement (σ^2), and efficiency may therefore be expressed in terms of variance per unit length. Plate height (H) is then given by the equation:

$$H = \frac{s^2}{L} \qquad (2.3)$$

where L is the length of the column and σ^2 carries units of length squared. Plate height therefore represents a linear distance. The plate height may be considered as the length of column that contains the fraction of solute that lies between L – σ and L. The column therefore becomes more efficient with smaller values of H, which implies that the column can generate more concentrated solute bands [2]. The plate count (N) is related to H by the equation:

$$N = \frac{L}{H} \qquad (2.4)$$

where L is the length of the column packing. Plate count can be calculated experimentally by determining $W_{1/2}$, the width of the peak at half-height (which is also defined as 2.354 × σ). N is then given by:

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \qquad (2.5)$$

The efficiency of a chromatographic column increases as the plate count becomes greater. Plate count and plate height are used to compare efficiencies of different columns by using the same compound to measure these parameters [2].

Peak broadening occurring during the chromatographic separation, on-column peak broadening, is the consequence of several factors. The contribution of each of these processes to the plate height is described by the Van Deemter equation:

$$H = A + \frac{B}{u} + (C_S + C_M).u \qquad (2.6)$$

where u is the linear velocity of the mobile phase and the coefficients A, B and C are related to the phenomena of multiple flow paths, longitudinal diffusion and mass-transfer between the phases, respectively. C_S and C_M are mass-transfer coefficients for the stationary and mobile phases, respectively [2]. Figure 2.1 graphically relates the contribution of each of these factors to H.

The multi-path term (A) describes peak broadening that results from the multitude of pathways by which a solute molecule can find its way through a packed bed. Due to the variable lengths of these pathways, the residence time in the column for molecules of the same species differs, leading to peak broadening. This effect, also called eddy diffusion, is directly proportional to the diameter of the packing particles. Smaller particles and narrow particle-size distribution reduce the contribution of the A-term to peak broadening. Multi-path peak broadening may also be partially offset by ordinary diffusion, which results in the transfer of molecules between streams following different pathways. At low linear velocities, numerous pathways are sampled by each molecule and the rate at which each molecule moves down the column tends to approach the average [2,4].

The longitudinal diffusion term (B/u) describes band broadening due to the diffusion of solute molecules in the mobile phase (i.e. from the concentrated center of the band to the more dilute regions ahead and behind it). The longitudinal diffusion term is directly proportional to the diffusion coefficient of the species in the mobile phase, D_M, as well as to the concentration difference (between the center of the band and the more dilute regions ahead and behind it), and inversely proportional to the mobile phase velocity [2,9].

Band broadening resulting from mass-transfer effects arises because the many flowing streams of mobile phase within the column and the layer making up the stationary phase both have finite widths. Consequently, time is required for solute molecules to diffuse from the interior of these phases to the phase interface where distribution occurs. This time lag results in the persistence of non-equilibrium conditions along the length of the column. The mass-transfer effect on plate height is related to the square of particle size and to the velocity of the mobile phase since long diffusion distances and fast flow rates leave less time for equilibrium to be approached [2,9].

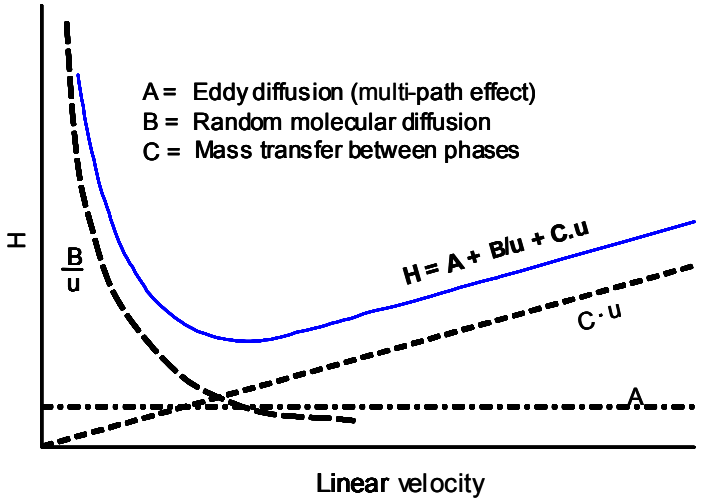


Figure 2.1. The contributions of A, B and C-terms to the plate height, H, in a packed column.

2.5. Optimisation of chromatographic resolution

A chromatographic separation is typically optimised by varying experimental conditions until the components of a mixture are separated efficiently in the shortest time. Resolution (R_s) can be expressed in terms of N or H, α and k (equation 2.2) and each of these factors can be manipulated to optimise R_s. Optimisation of α has the largest effect on R_s. Selectivity is optimised by changing the stationary phase or the mobile phase in LC [2].

The effect of k on R_s is small for values above 5, whereas low k values result in poor R_s. In chromatographic separations, one of the main objectives is often adequate R_s (≥ 1.5) in the shortest time. In the separation of multi-component mixtures, which contain solutes of widely varying distribution constants (resulting in a wide disparity in retention factors), this objective may not always be possible with an isocratic mobile phase – a phenomenon commonly referred to as the *general elution problem*. In LC, variations in k can be introduced during elution by dynamically changing the composition of the mobile phase – a technique known as gradient elution [2]. Most current LC separations are performed in gradient mode to benefit from increased speed and efficiency. N (equation 2.5) is not a valid measure of column efficiency when gradient elution is performed as peak widths and retention times are altered dynamically throughout the separation. The resolving power of a gradient separation is better expressed in terms of peak capacity (n_p), defined as the number of peaks that can theoretically be separated with a given resolution in a given time. Peak capacity can be calculated using the following equation:

$$n_p = \frac{\sqrt{N}}{4R_s}.\ln\frac{(1+k_1)}{(1+k_f)}+1 \qquad (2.7)$$

where k_1 and k_f are the retention factors for the first and last peaks, respectively, and R_s is the required resolution between each pair of successive peaks [2,8].

Resolution can also be optimised by increasing N or reducing H. Note that R_s is proportional to the square root of N, so that a fourfold increase in N doubles R_s. Plate number can be increased by using longer columns, thereby incurring increased separation time, peak broadening and operating pressure. Plate height may be decreased by reducing the particle size (at the cost of higher operating pressures) and operating at the minimum of the van Deemter curve [2]. It should be noted, however, that for a given operating pressure, higher maximum efficiencies can be obtained on columns packed with larger diameter particles, as this will allow the use of longer columns (higher N), but incurring longer analysis times. The optimal particle size for a given application will therefore depend on the maximum pressure, required efficiency and available analysis time [1-3,7,8,10,11]. Due to the reduction in resistance to mass transfer realised by small-particle columns, the latter may be operated at higher linear velocity without appreciable loss in efficiency. Therefore, the use of small-particle columns results in faster, more efficient separations, although the price to pay is in terms of higher operating pressures [12]. The effect of particle size on efficiency and optimal mobile phase velocity is demonstrated in Figure 2.2 [12]. The use of small (sub-2 µm) particle-packed columns operated at elevated pressures (> 400 bar) is referred to as UHPLC.

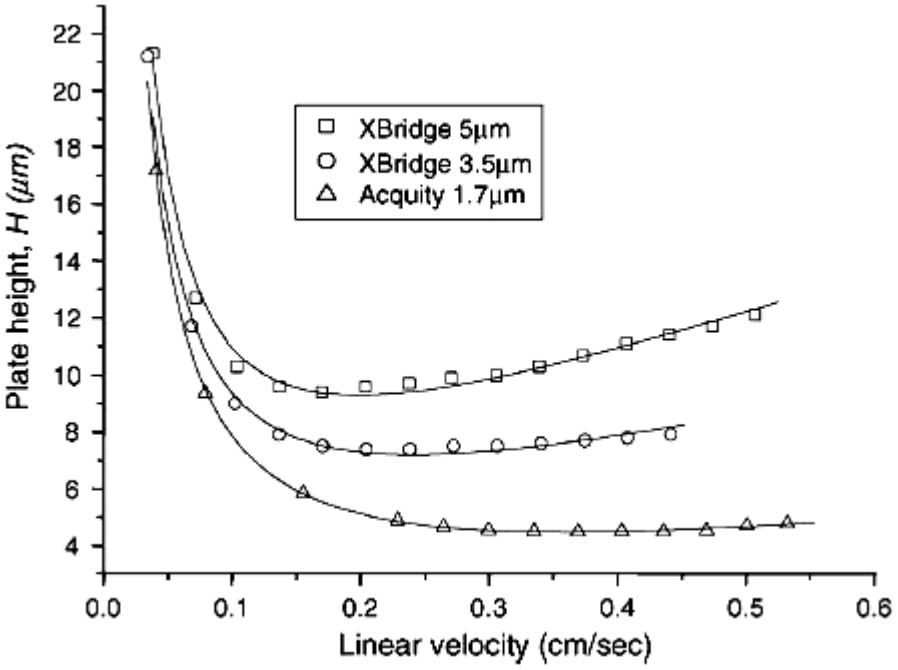


Figure 2.2. The effect of particle size on efficiency and mobile phase velocity in HPLC [12].

Column efficiency in HPLC is theoretically independent of diameter, but may be affected by packing homogeneity and quality. Reducing the diameter of the column facilitates rapid dispersion of the heat generated as a result of resistance to flow experienced in small-particle columns, an important consideration in UHPLC [13,14]. This frictional heating is important, since diffusivity of the solute in both phases, the viscosity of the mobile phase and the solute distribution coefficients are temperature dependent. It follows that a consistent column temperature profile reduces peak spreading [7]. Moreover, reducing the internal diameter of the column results in lower optimal flow rates and therefore significantly reduced solvent consumption. This results in small peak volumes compared to larger-diameter columns (for equal injections). However, maximum sample size is directly proportional to column volume so that an optimally sized injection will yield identical peak concentrations in small and large diameter columns, respectively.

In addition to UHPLC, other recent approaches to improve HPLC performance include the development of superficially porous stationary phase materials and advances in high temperature liquid chromatography (HTLC). Pellicular (or superficially porous) packing materials use solid core particles with porous surface chemical modification to yield smaller diffusion distances. A reduction in the flow-through pore size improves the mass transfer properties of the material [4,10]. High temperature liquid chromatography uses elevated temperatures to reduce the mobile phase viscosity, resulting in improved mass transfer and reduced operating pressures. Mobile phase pre-heating is of critical importance in HTLC in order to prevent excessive peak broadening due to radial temperature gradients inside the column. For example, Guillarme *et al.* demonstrated that it is possible to achieve significant increases in the speed and efficiency when operating at 200°C on a column of 1 mm internal diameter [15].

2.6. Modes of separation in liquid chromatography

The basic process of retention in LC is the result of distribution of solutes, on a molecular level, between the two phases. In LC, the solutes interact with the stationary phase as well as the mobile phase: modes of interaction include liquid–solid, liquid–liquid, ion exchange and size exclusion chromatography [2,7]. The nature and magnitude of solute interactions with the two phases controls retention. The exception is size exclusion chromatography, where pore size exclusively controls retention.

In other modes of separation, pore size has a limited effect on retention through controlling access of the solute to the stationary phase. The basic types of molecular forces involved are ionic forces, polar forces (including hydrogen bonding) and dispersive forces. It should be noted that in most distribution systems combinations of these forces are present and selectivity is not exclusively the result of one mechanism, but rather the result of the dominant force and secondary interactions [7]. These fundamental liquid chromatographic separation modes will be discussed with reference to the three basic molecular forces involved.

Dispersive forces are electric in nature, but result from charge fluctuations rather than permanent electric charges on molecules, for example the molecular forces that exist between hydrocarbon molecules. Selective retention of solutes on the basis of dispersive interactions requires the stationary phase to contain only hydrocarbon-type materials, whereas the mobile phase must be polar or significantly less dispersive. These are known as reversed phase (RP) separations, the most widely used separation mode in liquid chromatography [7]. Retention occurs by non-specific hydrophobic interactions of the solute with the stationary phase and it involves mainly apolar solutes or apolar portions of molecules. Optimum retention and selectivity is most likely where the solutes have a predominant aliphatic- or aromatic character and limited hydrogen-bonding groups. Hydrophobic retention is reduced by increasing the fraction of organic solvent in the aqueous mobile phase - the less polar the added organic solvent, the greater the effect [6]. The predominant factors that determine the hydrophobicity of the stationary phase are the length of the alkyl chain attached to the silica support, the total number of carbon atoms as well as the bonding density [2,3]. Solute-solvent interactions, such as solubility effects, are critical in reversed phase chromatography as solute interactions with the stationary phase are relatively weak, non-specific dispersive interactions. The popularity of reversed phase liquid chromatography (RP-LC) is due to its unmatched simplicity, versatility and scope. The near universal application of RP-LC stems from the fact that virtually all organic molecules have hydrophobic regions in their structure and are capable of interacting with these stationary phases, while rapid equilibration of the stationary phase with changes in mobile phase composition ensures amenability with gradient elution [4].

The stationary phase in RP-LC is commonly obtained by chemical derivitisation of silica particles with alkyl moieties such as C18 functional groups or phenyl groups. The hydrocarbon is attached to silanol groups on the silica support particles *via* covalent bonds and these bonded-phase packings are mechanically stable compared to liquid stationary

phases [2]. For steric reasons, it is not possible for all silanol groups to react and consequently a small percentage of un-derivatised silanol groups remain on the surface. Remaining silanol groups may be inactivated by reaction with a suitable silylating agent that is able to penetrate the location of the unreacted silanol groups. This process, known as endcapping, renders the material less polar, by reduction of possible secondary polar interactions. The additional polar and ionic interactions provided by silanol groups in non-endcapped phases may enhance selectivity where the solute posses some polar character, but often also cause unwanted band broadening for basic compounds. The main limitation of silica as a support material is the pH range over which it is stable. Most chemically modified silicas are useful from pH ~2 to 8 and will experience accelerated degradation outside this range.

Polymeric materials possess wide pH stability, and when chemically modified with hydrophobic functional groups, for example polystyrene-divinylbenzene phases, may be used for RP separations. The possibility of utilising π–π interactions or charge transfer effects with phenyl phases leads to different selectivities on these phases. The large surface area associated with the polymeric sorbents imparts a relatively high capacity to the phase, although the tendency of the material to expand and contract in different mobile phase compositions often leads to non-reproducible chromatographic performance [2,3].

Sample focusing is a technique often used in RP-LC, where an injection solvent that is a significantly weaker eluent than the mobile phase is used to dissolve the sample. Focusing then occurs at the head of the column as the retention of the solutes is increased under these conditions. This technique is readily adaptable to RP-LC using an injection solvent such as water. Chromatographic efficiency is enhanced, with subsequent separation of the sample starting from a narrow, concentrated band [3].

Polar interactions arise from permanent or induced dipoles in molecules such as alcohols, ketones and aldehydes, or polarisable compounds such as aromatic hydrocarbons. To selectively retain polar molecules the stationary phase must also be polar, or when the solute is strongly polar, a polarisable substance may function as the stationary phase. However, to maintain strong polar interactions between the solute and the stationary phase, the mobile phase must be relatively non-polar or dispersive in nature. This mode of separation is known as normal phase liquid chromatography (NP-LC) [7]. Normal phase liquid chromatography makes use of inorganic adsorbents or polar functionalised bonded stationary phases (most commonly based on silica gel) and non-polar, non-aqueous mobile

phases. In these systems retention may be envisaged as competitive partitioning of adsorbed mobile phase molecules on the adsorbent surface by the solute. Solute retention can be tuned effectively, and almost exclusively, by varying the composition of the mobile phase. Binary solvent mixtures offer additional selectivity fine-tuning by varying the dipole, proton acceptor and proton donor forces [3,4].

Hydrophilic interaction chromatography (HILIC) uses a polar stationary phase (such as non-modified silica) and an aqueous-organic mobile phase to retain highly polar and ionisable solutes [6]. The stationary phase adsorbs a layer of water (or another polar solvent), rendering it more hydrophilic than the mobile phase, with the result that polar solutes preferentially interact with the stationary phase [16]. Due to its aprotic nature, acetonitrile is often used as the organic fraction in the mobile phase as this encourages stronger hydrogen bonding between solutes and the polar-adsorbed layer on the surface of the stationary phase. HILIC retention mechanisms are an intricate multi-modal combination of liquid-liquid partitioning, adsorption, ionic interactions and hydrogen bonding. Retention is regulated by the composition of the mobile phase (including factors such as pH and ionic strength), its interaction with the stationary phase as well as the chemical properties and structure of the solute [17]. HILIC is therefore viewed as an aqueous variant of NP-LC as retention is proportional to the polarity of the solute and inversely proportional to the polarity of the mobile phase [17]. Normal phase liquid chromatography, which is also used to separate polar solutes, is inherently incompatible with electrospray ionisation (ESI) in LC-MS [1,18]. HILIC therefore complements RP-LC since solutes elute with increasing polarity and it is inherently compatible with ESI-MS detection. The acetonitrile-rich mobile phases typically used in HILIC separations provide conditions that are particularly favorable for efficient droplet formation and desolvation in ESI sources, typically leading to improved sensitivity compared to RP conditions in LC-MS applications [17].

2.7. Liquid chromatography – mass spectrometry instrumentation

2.7.1. The liquid chromatograph

The modern LC system is a very complex device designed to support its most critically important component, the column. Its development is the direct result of practical application of LC column theory [7]. Low solute diffusion and high mobile phase viscosity practically limit LC to packed columns, where small particles are exploited to reduce diffusion distances. The evolution of LC columns has resulted in columns packed with particles of ever decreasing size (and column diameters), resulting in significant increases in speed and/or efficiency. These columns require increasingly sophisticated instruments capable of operating at high

pressures to fully exploit the benefits offered by small-particle columns and to minimise extra-column band spreading [2-4,19]. Band spreading occurs in the column as well as in the void volume of the connecting tubing, injector and detector. In these extra-column volumes, band spreading results from the typical parabolic velocity profile of the mobile phase. Band spreading also results from the fact that solute in any dead volume is not displaced cleanly by the advancing mobile phase, but is rather eluted at a solute concentration which decreases logarithmically with time. It therefore follows that injection devices and detector cells need to be reduced in volume and that connecting tubing needs to be minimised so that their effect on column performance is negligible. Columns of 4.6 mm internal diameter used in most current HPLC instruments generate sufficiently large peak volumes to negate extra-column peak dispersion in these systems. However, as the column radius is reduced, peak volumes become smaller, and demands on the dispersion characteristics of all components of the LC system increase [4,14,19-22]. The latest advances in LC have produced UHPLC technologies designed specifically for maintaining the resolution achieved with highly efficient (small-particle), small-diameter columns.

2.7.2. Detectors for liquid chromatography

The ideal detector for LC should be sensitive and selective, and characterised by a linear response to solute concentration over a wide dynamic range. Furthermore, the detector should be reliable, with good stability and reproducibility, non-destructive, and have a small internal volume (to reduce extra-column band broadening). To be compatible with modern highly efficient, small-particle columns, the detector should also have a fast response time [2,19].

The most common LC detectors in use are based on UV/vis absorption. Diode array detectors are the most powerful UV/vis spectrophotometric detectors and permit simultaneous collection of data over a wavelength range of approximately 190 to 900 nm. Diode array detectors work in a parallel configuration, by simultaneously monitoring all wavelengths. Energy from the flow-cell is focused onto a dispersion device, typically a grating, and the resulting monochromatic wavelengths are directed onto an array of photodetectors, so that complete spectra can be obtained in fractions of a second [3,4]. Absorption by molecular oxygen limits the range of conventional UV/vis detectors to wavelengths longer than approximately 190 nm [23,24].

2.8. The mass spectrometer

Improvements in the efficiency of LC columns have led to the separation of increasingly complex mixtures, resulting in a demand for identification techniques linked to the LC. Several spectroscopic detection systems have evolved from this requirement, such as LC coupled to diode array UV/vis spectrometers (LC-DAD), fluorescence detectors (LC-FLD), Fourier transform infrared spectrometers (LC-FTIR), nuclear magnetic resonance spectrometers (LC-NMR) and mass spectrometers (LC-MS) [2,4,14]. In principle, LC is one of a number of sample introduction techniques for MS, but tandem application with chromatography offers much additional value such as selectivity and convenient quantitation [6].

Mass spectrometry is one of the most widely applicable analytical tools as it can be used to obtain qualitative and quantitative information about the atomic and molecular composition of inorganic and organic materials. The main advantages of MS are increased sensitivity and specificity compared to most other analytical techniques. The sensitivity and specificity results primarily from a combination of the analyser functioning as an effective mass-to-charge (*m/z*) ratio filter (thereby reducing background interference), sensitive electron multiplier detectors and characteristic fragmentation patterns of solute molecules [3,4,6]. For these reasons, the mass spectrometer is probably the closest to the ideal detector currently available for LC. Functionally an MS performs three primary tasks, namely conversion of the target solutes to gaseous ions, separation of the ions in vacuum according to their *m/z* ratio and detection of the separated ions [4].

2.8.1. The LC-MS interface

The fundamental challenge in coupling LC with MS is the enormous mismatch between the relatively large mass flows involved in LC and the vacuum requirements of MS [2,4,6]. The development and commercialisation of atmospheric pressure ionisation (API) mass spectrometry has led to the evolution of LC-MS into a sensitive, rugged and versatile technique [5,6]. Atmospheric pressure ionisation techniques such as ESI and atmospheric pressure chemical ionisation (APCI) are used almost exclusively in current LC-MS interfacing. In these API techniques the column effluent is nebulised and ionisation takes place in the aerosol as the eluent is removed, either with or without an external source of ionisation, followed by introduction of the ions into the high-vacuum environment of the MS [6]. Most API interfaces use nitrogen as curtain and nebulisation gas [6]. Ionisation of target compounds is achieved by spraying the eluent either from an electrically charged capillary or across a coronal discharge needle during the final stages of evaporation. The configuration

may be changed to produce positively or negatively charged ions. Ions are moved electrostatically into the entrance chamber of the MS assisted by movement from atmospheric pressure to high-vacuum. Most API techniques typically utilise off-axis flow paths to ensure that only charged species enter the mass spectrometer, while mobile phase solvent is diverted out of the system [1,4,6]. A specific API technique may fail to ionise some compounds and switching between sources is then required. Multimode sources capable of ionising diverse compounds are available, although this design suffers from a loss in sensitivity compared to dedicated sources [1].

In API ionisation it is important to consider the solution chemistry in order to optimise performance. For most solutes ESI response is primarily determined by liquid-phase chemistry, whereas in APCI the response is determined by gas-phase chemistry. Conditions such as solvent choice, pH and flow rate need to be optimised to enhance factors such as the formation of ions in solution, nebulisation, desolvation and ion evaporation. Consequently, the LC separation must often be adjusted to be suitable for LC-API-MS. For example, typical LC additives such as phosphate buffers are not suitable and must be replaced by volatile mobile phase additives. In RP-LC protolysis of solutes that show liquid-phase acid-base behaviour is avoided and buffering or ion-suppression are generally required for their separation. In contrast, ESI generally requires pre-formed ions in solution and is therefore incompatible with these separations. In RP-LC-ESI-MS the chromatographic parameters (such as stationary phase) may be changed so that the organic modifier content is maximised to enhance ionisation, while HILIC may be used to achieve this effect for separation of polar solutes. Adduct formation is often observed for solutes that show an affinity for sodium. This phenomenon may be exploited in instances where the response of the sodiated ion is better than that for the molecular ion. Alternatively, ammonium acetate can be used to direct adduct formation consistently towards a single species, rendering the process suitable for quantitative analysis. Post-column techniques may also be used to decouple LC separation and API detection requirements for optimal performance of both components [5,6].

In LC-MS quantitation is often complicated by matrix effects, i.e. suppression or enhancement of analyte response due to co-eluting sample matrix components. It has been demonstrated that matrix suppression is primarily a liquid-phase rather than a gas-phase process, and that it mostly involves non-volatile elements that prevent pre-formed solute ions from transferring to the gas phase. Ionisation suppression is therefore mostly associated with ESI. Matrix effects can be removed by improving sample pre-treatment and/or

chromatographic separation. Alternatively, matrix effects can be avoided by changing from ESI-MS to APCI-MS, switching from positive to negative ionisation (or *vice versa*) or using a suitable a mobile phase additive. Quantitation in the presence of matrix suppression can also be performed by using an isotopically-labeled internal standard or matrix-matched calibration standards. Selection of a suitable internal standard for multi-residue methods is, however, difficult as an isotopically-labeled or analogue internal standard may not produce valid results for diverse target solutes. When appropriate blank materials are not available for preparation of matrix-matched calibration standards, the standard addition method, although time-consuming and laborious, can be used for accurate and precise quantitative results [6].

Atmospheric pressure ionisation spectra principally furnish information regarding the molecular weight of the solute (the molecular ion is most often the base peak) and do not provide the same level of structural information as for example electron impact ionisation (EI), which is most frequently used in GC-MS [14]. In LC-MS, fragmentation of target product ions is primarily produced *via* collision induced dissociation (CID), either in the ion source or in dedicated collision cells such as in triple quadrupole mass spectrometers [6].

2.8.1.1. Electrospray ionisation

In the ESI interface the column effluent is nebulised into an API source while a high electric field is applied between the column exit capillary and a surrounding counter electrode. The effluent is converted into small charged droplets by a combination of the strong electric potential and high speed, heated concurrent nitrogen flow. As the neutral solvent molecules evaporate from the droplet surface, the size of the droplet is reduced, resulting in reduction in the distances between excess charges at the surface. This process continues until the surface tension of the liquid can no longer accommodate the increasing Coulomb repulsion between excess charges. A Coulomb explosion then disintegrates the droplet; repetition of this process leads to successive formation of ever decreasing droplet sizes, until gas phase ions remain. The gas phase ions are attracted towards a capillary sampling orifice through which they pass into the low-pressure region of the ion source [6].

The production of solute ions from the charged droplets is mainly the result of three processes at the droplet surface (soft desolvation, ion evaporation ionisation and chemical ionisation) or by gas-phase ion-molecule reactions. The soft desolvation and ion evaporation ionisation models require pre-formed analyte ions in solution. This is accomplished by appropriate control of mobile phase pH for basic and acidic solutes, respectively.

Electrospray ionisation may therefore be described as mixed-mode ionisation, since various processes contribute to the final result [1,6]. Electrospray ionisation is most often applied in combination with RP-LC as an electrically conductive mobile phase is required for effective charge transfer. In general, higher ESI ionisation efficiencies are obtained at lower flow rates, a phenomenon ascribed to smaller droplets which enhance the transfer of ions in solution to the gas phase due to improved surface-to-volume ratios. Relatively low mobile phase flow rate requirements make this technique particularly suited for use with small-diameter columns (and often requires effluent splitting for larger diameter columns) [4,6]. Figure 2.3 shows a graphic representation of the pneumatically assisted ESI source and ionisation process.

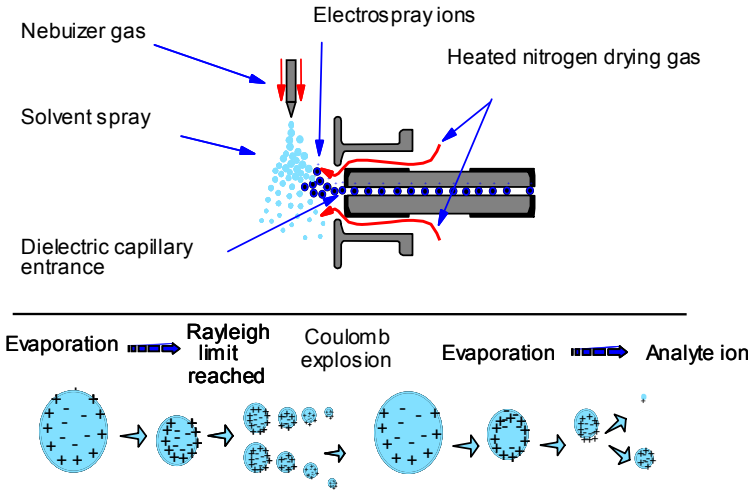


Figure 2.3. Pneumatically assisted electrospray interface and schematic ESI process.*

* Agilent Technologies Inc., Waldbronn, Germany.

2.8.1.2. Atmospheric pressure chemical ionisation

Atmospheric pressure chemical ionisation (Figure 2.4) utilises a heated, inert nebulising gas to entrain and break up the eluent stream into small droplets which are sprayed across a corona discharge needle. After desolvation, a dry vapour of solvent and analyte molecules is produced. Solvent molecules are ionised by electrons produced in the corona discharge and act as an ionised reagent gas to ionise analyte molecules by chemical ionisation. Typical gas-phase ion-molecule reactions comprise proton transfer, charge exchange, electrophilic addition as well as anion abstraction (positive ionisation) and proton transfer in negative ionisation. Atmospheric pressure chemical ionisation may therefore be described as solvent-

mediated chemical ionisation *via* ion-molecule reactions. Ions enter the low-pressure region of the MS through an orifice charged oppositely to the corona needle. A counter-current flow of dry nitrogen gas acts as a curtain to sweep uncharged solvent vapours away from the pinhole orifice, thus minimising clustering of charged analyte ions with water and other polar molecules. Atmospheric pressure chemical ionisation is typically used for less polar compounds and can accommodate relatively large flow rates, and it is compatible with pure organic and apolar mobile phases typically encountered in NP-LC and HILIC [1,6].

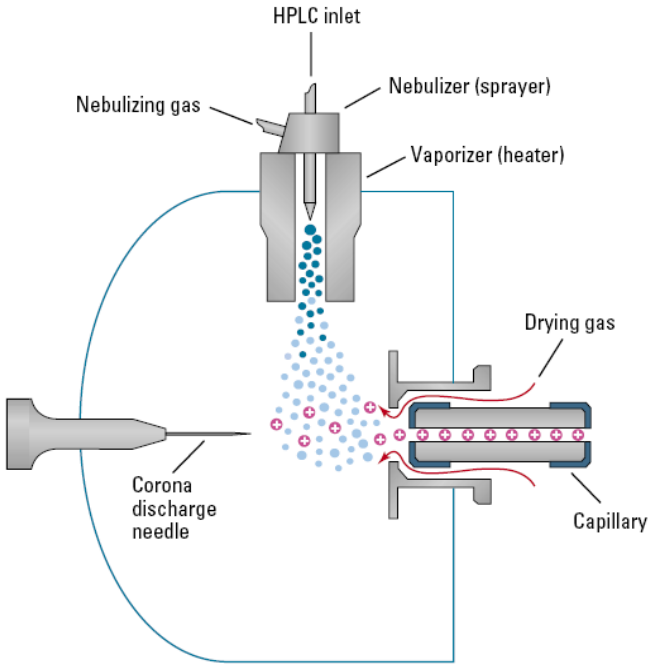


Figure 2.4. Atmospheric pressure chemical ionisation interface and schematic APCI process.*

* Agilent Technologies Inc., Waldbronn, Germany.

2.8.2. Vacuum system and ion optics

Atmospheric pressure ionization interfaces are gas-phase analyte enrichment systems since ions created in the spray chamber are preferentially introduced into the vacuum system. The ions are moved from the interface through a pinhole entrance and skimmer (typically orthogonally positioned relative to the sprayer) into the first-stage low-pressure region of the mass spectrometer. In most mass spectrometers the vacuum system consists of differentially pumped vacuum regions, evacuated by a mechanical fore-pump assisting high-vacuum turbomolecular pump(s) [6].

2.8.3. The mass analyser

The most important characteristic of a mass analyser is its resolving power (Figure 2.5). Mass peaks of ions have no natural line width so that the breadth of a peak is characteristic of the mass analyser performance. Recorded ion peaks are Gaussian in shape and resolution is expressed as full width at half of the maximum height (FWHM) of the peak in the profile mass spectrum. For a singly-charged ion the resolution (R) is given by the following equation:

$$R = \frac{(m/z)}{FWHM} \qquad (2.8)$$

A quadrupole analyser is typically operated at unit-mass resolution with a FWHM of ~ 0.6 u for a singly-charged ion. Analysers that are capable of very high resolution can be used to measure the mass of an ion with sufficient accuracy to determine its atomic composition from the known theoretical atomic masses [3,6].

The primary function of all mass analysers is the separation of ions according to their *m/z*, either in time or in space. Mass spectrometry may be performed in any of two general data acquisition modes, namely full-spectrum analysis or selected-ion monitoring (SIM). In full-spectrum analysis mode, the amount of each mass unit is measured continuously throughout the experiment, over a defined mass range. A total ion current profile is generated in this manner that represents a normalised plot of the sum of ion abundances as a function of time. Mass spectra are obtained for each of the sequence of scans thus performed. Full-spectrum analysis mode is principally used for compound identification and structure elucidation studies. In targeted analysis (SIM mode), data can be acquired for one or a few ions or fragments of ions, to produce a signal that is very specific to the targeted compound. As fewer mass measurements are made than in scan mode, the measurements can be repeated more often, resulting in a proportional increase in sensitivity due to elimination of noise through averaging of the signal.

Liquid chromatography – mass spectrometry systems are categorised into four basic designs according to the mass analyser system used, namely the quadrupole (or octapole) type analysers, ion trap analysers, time-of-flight analysers and Fourier-transform ion-cyclotron instruments. Combinations of two or more of these designs are used to create hybrid systems that combine multiple mass analyser modules with collision and ion-trapping cells to separate, fragment and detect not only the molecular ion, but also fragments of ions [1,3,4,6].

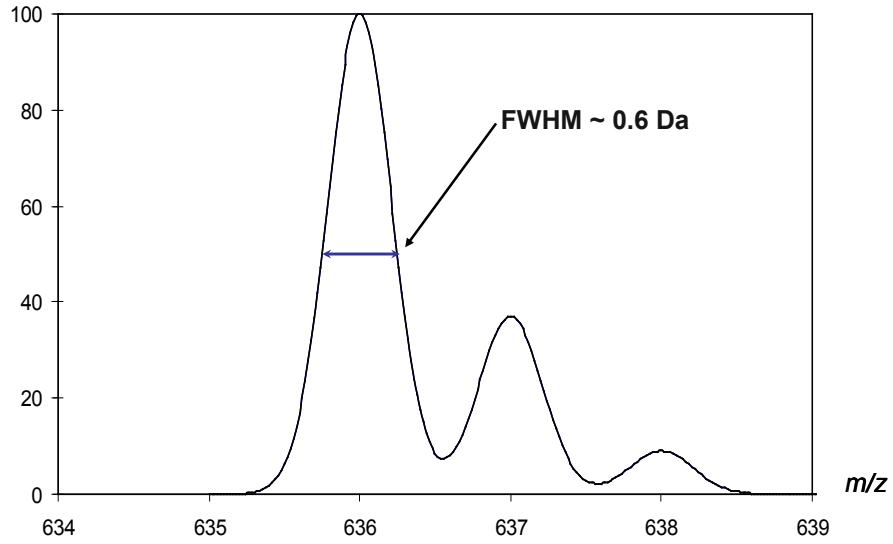


Figure 2.5. Schematic representation of a mass spectrum recorded at unit mass resolution. *

* Waters Corporation, Milford, U.S.A.

2.8.3.1. Linear quadrupole mass spectrometry and tandem mass spectrometry instruments

The linear quadrupole (or octapole) type mass analyser is the most widely applied detector in LC-MS. It uses a parallel bundle of oppositely charged rods that are placed in a radial array. Opposite rods are charged by a positive or negative direct-current (DC) potential, while adjacent rods have opposing charge. An oscillating radiofrequency alternating-current (RF) voltage is superimposed over the arrangement so that the latter successively reinforces and overwhelms the DC field. Ions are introduced into this quadrupole field *via* a low accelerating potential. At a given combination of DC and RF voltages applied to the rods, the trajectories of ions of a particular *m/z* are stable and these ions oscillate in a plane perpendicular to the rod length. These ions traverse the quadrupole filter following a corkscrew flight path as they are swept forward by the RF signal and are transmitted to the detector. Ions of other *m/z* have unstable trajectories and as the amplitude of their oscillations become infinite, they are discharged on the rods and/or become lost in the vacuum. In scan mode, ions in a predetermined *m/z* range are consecutively transmitted towards the detector by sweeping the DC and RF potentials at a constant ratio. The resolution of a quadrupole is determined by the ratio of DC and RF as well as the quality and alignment of the rods. Enhanced resolution leads to a significant loss in response; most quadrupole analysers are operated at unit-mass resolution [1,4,6].

Structure elucidation problems often require more information than may be obtained from the API-based soft-ionisation processes, since these typically provide mainly molecular ion information. Fragmentation of an even-electron ion can be induced *via* CID to yield various product ions. In CID, collisions with neutral gas molecules are used to convert ion translational energy into internal energy, which leads to subsequent unimolecular decomposition. In API-based instruments CID may be achieved by increasing the potential difference between the ion-sampling orifice and skimmer in the ion source. However, such in-source CID produces fragmentation of all ions entering this region and offers no pre-selection of precursor ions.

Triple quadrupole tandem mass spectrometry (MS/MS) instruments combine two conventional scanning quadrupole analysers separated by a collision cell. The collision cell is a RF-only quadrupole that can function as an ion guide, or when filled with gas, as a collision cell. The energy of these collisions, and therefore the degree of fragmentation, is regulated by the collision voltage. A selected target ion from the first analyser is allowed to collide with inert gas molecules in the collision cell to induce fragmentation. The fragmented ions are then passed into the second analyser for mass analysis and subsequent detection. A stacked-ring RF ion-transmission device replaces the RF-only quadrupole in some analysers [1,4,6]. To identify these components in the following discussion, the first quadrupole analyser, collision cell and second quadrupole analyser will be numbered Q1, Q2 and Q3, respectively.

The triple quadrupole LC-MS (Figure 2.6) can be operated in any one of four modes depending on the aim of the experiment. In the precursor-ion mode, Q1 is scanned and all ions sent to the collision cell. Quadrupole Q3 is then tuned at a frequency to select a specific fragment ion common to related compounds. In the product-ion mode, Q1 is fixed at a suitable frequency to select a specific ion that is passed to the collision cell. Quadrupole Q3 is then scanned for fragmentation information that can be used to identify the structure of the ion under investigation. In the neutral loss mode, both Q1 and Q3 are scanned with a specific frequency offset. Only ions that loose a common uncharged fragment are detected, thereby providing information about their fragmentation type and molecular weight. The fourth operational mode, known as selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), is particularly suited for trace analysis of compounds in complex mixtures, even in cases where the components are not completely separated. In this mode, Q1 and Q3 are set at ion frequencies specific for the compound under investigation and one or more of its product fragments, respectively. The signals that are generated are therefore

very specific to the target compound while interferences are excluded. By using a small scan range and a high signal sampling rate, a greater number of data points may be averaged for a given *m/z* value over time, thus producing a signal with higher signal-to-noise (S/N) ratios. In this way sensitivity and detection limits are optimised [1,3,6].

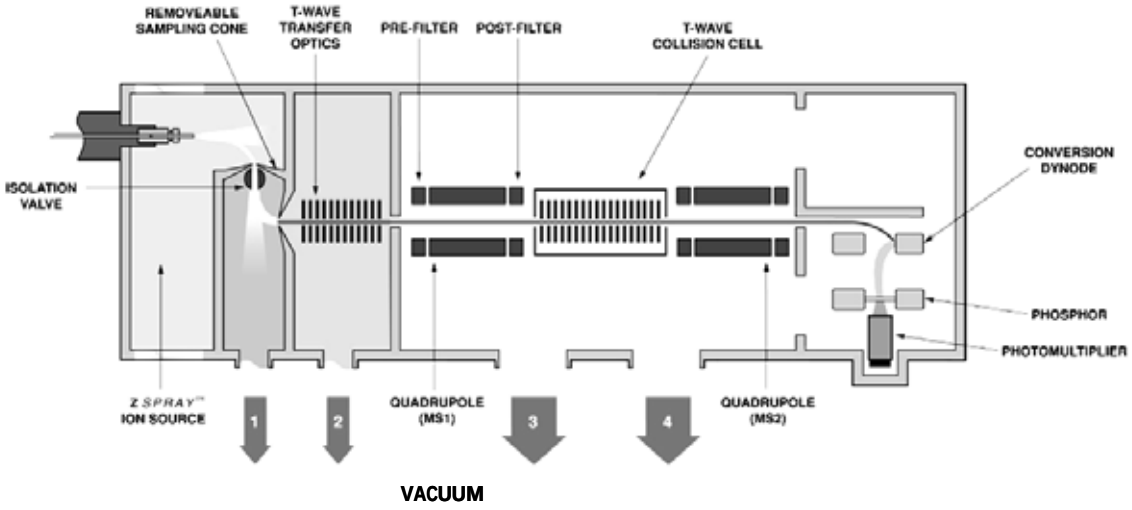


Figure 2.6. Schematic representation of a tandem quadrupole instrument illustrating the source, ion optics, mass analysers, ion detector and vacuum system. *

* Waters Corporation, Milford, U.S.A.

2.8.3.2. Time-of-flight instruments

In a time-of-flight (TOF) mass analyser (Figure 2.7) ions are accelerated in a pulsed mode into a field-free linear flight tube where the travelling time to the detector is dependent on the *m/z* of the ions, with lighter fragments arriving at the detector first. Pulsed ion introduction is required to avoid simultaneous arrival of ions of various *m/z* at the detector. Unlike quadrupole or ion trap designs, the TOF analyser does not use scanning for the acquisition of a mass spectrum. Rather, spectra from different ion introduction events are accumulated, resulting in improved S/N due to averaging of random noise. The TOF mass analyser also has a greater mass range compared to quadrupole or ion trap designs, with capabilities up to *m/z* 20 000. However, the mechanism of TOF measurements precludes SIM mode of operation [1,6].

Due to kinetic energy dispersion of ions leaving the ion source, the resolution achievable with the TOF design is limited by the length of the flight tube. Since improved separation between ions of differing *m/z* is obtained with a longer tube, an ion mirror (or reflectron) is often used to double the length of the flight tube without compromising the dimensions of the

instrument. Mass resolution may also be improved by minimising the initial kinetic energy spread of ions as they are introduced into the flight tube. This is typically achieved in API-LC-TOF-MS by accelerating the ions orthogonal to their direction of introduction into the flight tube. In this way the longitudinal kinetic energy spread is reduced, resulting in improved mass resolution (typically better than 10 000), which translates to higher mass accuracies (typically better than 2 parts per million) [1,6].

In order to perform MS/MS experiments, the TOF analyser has to be combined with another mass analyser in a hybrid system. The most successful of these is the quadrupole-time-of-flight (Q-TOF) instrument. In MS mode the quadrupole is operated in RF-only mode. Product-ion MS/MS may be performed when the quadrupole performs precursor-ion selection at unit-mass resolution followed by CID and mass analysis utilising the TOF device. In this way accurate-mass determination in both MS and MS/MS mode can be performed [6].

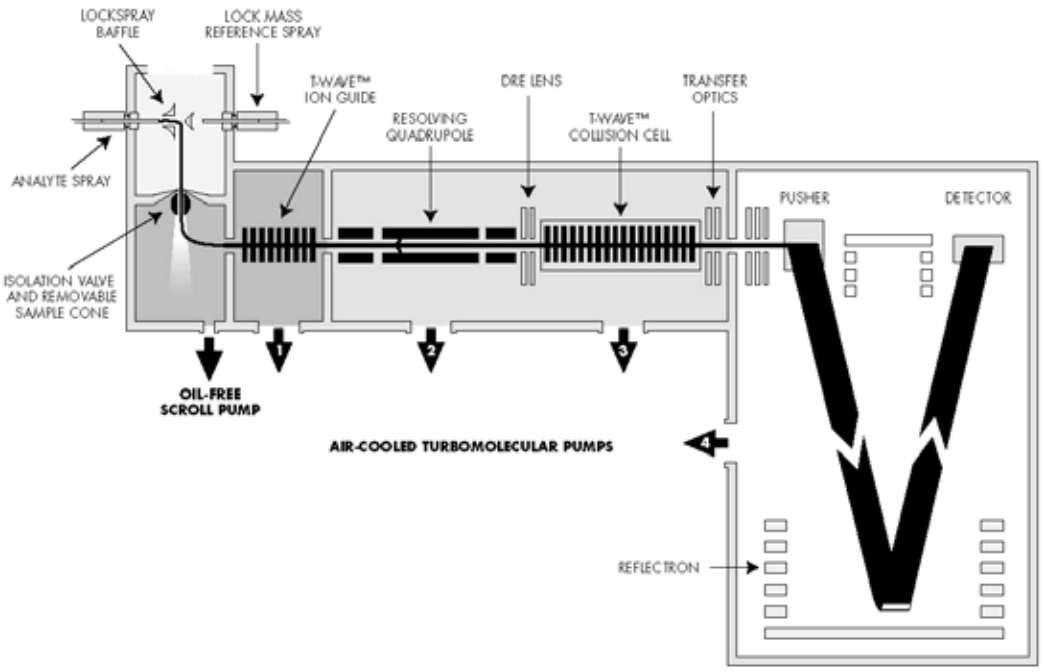


Figure 2.7. Schematic representation of the source, ion optics, mass analysers, ion detector and vacuum system of a quadrupole time-of-flight instrument. *

* Waters Corporation, Milford, U.S.A.

2.8.4. Ion detectors

Ions are expelled from the mass analyser as a function of *m/z*. The number of ions of each mass is recorded by directing them onto the electron multiplier which serves as an ion detector. Gain ranges of the order of 10^5 to 10^7 may be attained with this detector design [1,3].

2.9. Sample preparation for chromatographic analysis

Often in chromatographic analysis, the sample mixture is too complex, incompatible with the mobile phase, or too dilute to permit direct sample injection. In such instances preliminary extraction, fractionation, isolation and/or concentration of the sample are required. A variety of sample pre-treatment strategies may be applied for this purpose in combination with LC analysis. The specific sample pre-treatment strategy used is obviously dictated by the sample matrix as well as the target analytes and their concentrations. Sample pre-treatment strategies vary from elementary, such as dilution and filtration, through to highly efficient preparative LC separations.

2.9.1. Distillation

Distillation is suitable for isolation of volatile organic compounds from liquid samples or soluble solid samples. The efficiency of the separation is dependent on physical properties of the sample components and the method of distillation and equipment used. For example, a fractionating column facilitates contact between rising liquid vapours and returning condensed liquid so that more efficient separation can be achieved, while steam distillation is typically used to recover high boiling-point compounds. Ionic strength, pH and the addition of a co-distiller such as toluene, may be used to optimise the recovery of volatile organic compounds. Distillation is also an effective method for reducing large sample volumes as well as to facilitate subsequent sample preparation steps such as liquid extraction [4].

2.9.2. Liquid extraction and liquid-liquid extraction

Liquid-liquid extraction (LLE) is based on the selective partitioning of the solutes between two immiscible phases. Typically an aqueous solution is extracted with an immiscible organic solvent. Several variations of the technique are in use. Ion-pair extraction is a versatile and efficient method that is used to extract ionisable compounds such as acids, bases and aprotic ions such as quaternary ammonium ions. In solid-supported LLE the aqueous sample is applied to a dry bed of inert diatomaceous earth particles, which is eluted after equilibration with an immiscible organic solvent. In LLE quantitative recovery of the solutes depends on the distribution coefficients and phase ratios involved, and single or repeated

extraction steps may be used as appropriate. Exhaustive extraction is most conveniently performed using a separating funnel and a solvent of higher density than the aqueous solution, such as chloroform or dichloromethane, which may be renewed in a sequence of extractions. Selectivity is optimised by choice of extraction solvent and pH or ionic strength of the aqueous phase. Analyte enrichment can be achieved by subsequent solvent evaporation [4,6].

2.9.3. Solid phase extraction

Solid phase extraction (SPE) is a versatile sample preparation technique utilising a multitude of adsorbents for polar, hydrophobic and/or ion exchange interactions. Adsorbent chemistries and parameters for separation are based on the same principles that apply to equivalent LC techniques. The total sample capacity of these columns is approximately 1 – 5% of the sorbent mass, and since small volumes of solvent are typically used to elute target solutes, concentration factors up to 1000 can be achieved in favourable circumstances. Solid phase extraction can be used to establish three important pre-requisites for trace-level analysis, namely enrichment, removal of interfering matrix components and changing the matrix for subsequent analyses. Since the analyte can either be adsorbed on the SPE phase or flow through unretained, two general separation strategies are possible. In the first case (Figure 2.8), the liquid sample is forced through the conditioned cartridge where the analyte is retained on the phase. The matrix can then be washed off, followed by selective elution of the analyte. Alternatively, the conditioned phase may retain the interferents while the analyte passes through the column, allowing purification of the sample solution. Solid phase extraction offers several advantages over liquid extraction such as speed, a broad application range, low solvent consumption and potential automation (including on-line SPE-LC). Mixed-mode materials such as divinylbenzene-n-vinylpyrrolidone copolymers offer retention based on a combination of hydrophobic and ion-exchange interactions, imparting potential for additional selectivity optimisation to this phase [4,6]. In LC-MS, the overall degree of matrix elimination may be improved when retention mechanisms employed for sorbent extraction and analytical separation are complimentary, for example ion exchange SPE followed by RP-LC.

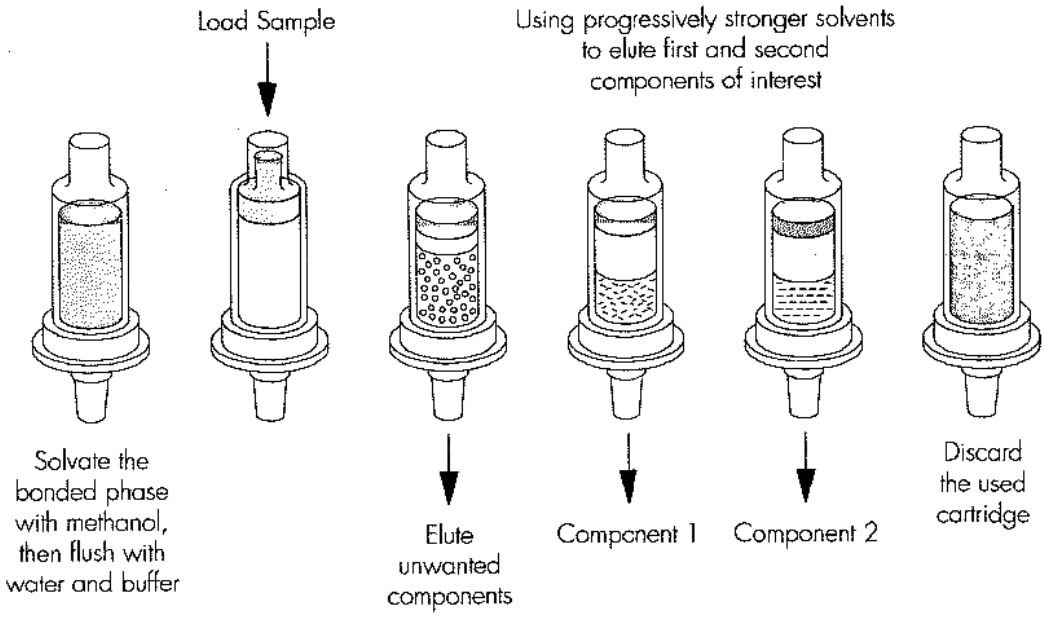


Figure 2.8. Schematic representation of the general reversed phase SPE elution protocol. *

* Waters Corporation, Milford, U.S.A.

References

[1] M.C. McMaster, LC/MS: A practical user's guide, John Wiley & Sons Inc., New Jersey, U.S.A. (2005).
[2] D.A. Skoog, F.J. Holler, T.A. Nieman, Principles of instrumental analysis, Saunders College Publishing, Philadelphia, U.S.A. (1998).
[3] H.H. Willard, L.L. Merritt, J.A. Dean, F.A. Settle, Instrumental methods of analysis, Wadsworth Publishing Company, California, U.S.A. (1988).
[4] C.F. Poole, S.K. Poole, Chromatography today, Elsevier Science Publishing Company Inc., New York, U.S.A. (1991).
[5] R.B. Cole, Electrospray ionisation mass spectrometry: Fundamentals, instrumentation and applications, John Wiley & Sons Inc., New Jersey, U.S.A. (1997).
[6] W.M.A. Niessen, Liquid chromatography – mass spectrometry, Taylor & Francis Group, Florida, U.S.A. (2006).
[7] R.P.W. Scott, Liquid chromatography column theory, John Wiley & Sons Ltd., West Sussex, U.K. (1992).
[8] P. Sandra, J. High Res. Chrom. 12 (1989) 82.
[9] P. Sandra, J. High Res. Chrom. 12 (1989) 273.
[10] G. Desmet, D. Cabooter, P. Gzil, H. Verelst, D. Mangelings, Y. Vander Heyden, D. Clicq, J. Chromatogr. A 1130 (2006) 158.
[11] H. Poppe, J. Chromatogr. A 778 (1997) 3.
[12] A. de Villiers, F. Lestremau, R. Szucs, S. Gélébart, F. David, P. Sandra, J. Chromatogr. A 1127 (2006) 60.
[13] A. de Villiers, H. Lauer, R. Szucs, S. Goodall, P. Sandra, J. Chromatogr. A 1113 (2006) 84.
[14] E. Heftmann, Chromatography: Fundamentals and applications of chromatographic and electrophoretic methods. Part A: Fundamentals and techniques, Elsevier Scientific Publishing Company, New York, U.S.A. (1983).
[15] D. Guillarme, S. Heinisch, J.L. Rocca, J. Chromatogr. A 1052 (2004) 39.
[16] D.V. McCalley, U.D. Neue, J. Chromatogr. A 1192 (2008) 225.
[17] E.S. Grumbach, K.J. Fountain, Comprehensive guide to hydrophilic interaction chromatography, Waters Corporation, Milford, U.S.A. (2010).
[18] R. Kostiainen, T.J. Kauppila, J. Chromatogr. A 1216 (2009) 685.
[19] F. Gritti, G. Guiochon, J. Chromatogr. A 1217 (2010) 7677.
[20] F. Gritti, I. Leonardis, D. Shock, P. Stevenson, A. Shalliker, G. Guiochon, J. Chromatogr. A 1217 (2010) 1589.
[21] S. Henisch, J-L. Rocca, J. Chromatogr. A 1216 (2009) 642.
[22] E.S. Grumbach, J.C. Arsenault, D.R. McCabe, Beginners guide to ultra-performance liquid chromatography, Waters Corporation, Milford, U.S.A. (2009).
[23] L.G. Wade Jr., Organic Chemistry, Prentice-Hall Inc., New Jersey, U.S.A. (1999).
[24] R. T. Morrison, R. N. Boyd, Organic Chemistry, Prentice-Hall Inc., New Jersey, U.S.A. (1992).

Chapter 3

Liquid chromatography – mass spectrometry in wine analysis: An overview

3.1. Introduction

Liquid chromatography (LC) is a versatile analytical technique that offers separations that are based on polarity, electrical charge and/or molecular size, and can be used to separate most mixtures that can be dissolved. Moreover, unlike gas chromatography (GC), LC is not limited by volatility or thermal stability of the analytes, and it is amenable to direct analysis of aqueous solutions [1]. Liquid chromatography – mass spectrometry (LC-MS) equipment is characterised by the type of mass analyser (MS) used and may be capable of MS or MS/MS (or indeed MS^n) operation (details of LC-MS equipment is given in Chapter 2). The advent of the hyphenation of MS to LC therefore created a very powerful analytical tool that has evolved to become the technique of choice in many areas of Analytical Chemistry. Critical attributes of this analytical technique are the determination of a wider range of analytes with higher sensitivity, selectivity and specificity, and LC-MS meets these criteria for many applications. It is therefore not surprising that LC-MS has found widespread application in wine analysis. The objective of this chapter is not to present an exhaustive review of the application of LC-MS in wine analysis, but rather an overview of the subject, discussed according classes of analytes.

3.2. Phenols and related derivatives

Phenolic compounds are a large and complex group of wine constituents that are extracted from the fruit and stems of the grape vine, some are products of yeast metabolism during fermentation, and others are derived from wood cooperage. Figure 3.1 shows the structures of representative examples of phenolic compounds.

Phenolics are of particular importance in determining the characteristics and quality of red wines, and also to a lesser extent those of white wines (where they occur in lower concentrations). Phenolic compounds influence the appearance, taste, mouth-feel, fragrance and anti-microbial properties of wine [2,3]. During winemaking and maturation the phenolic compounds participate in reactions that yield more complex compounds with different physical–chemical properties, thereby imparting important changes in colour and flavour properties to red wines in particular [4].

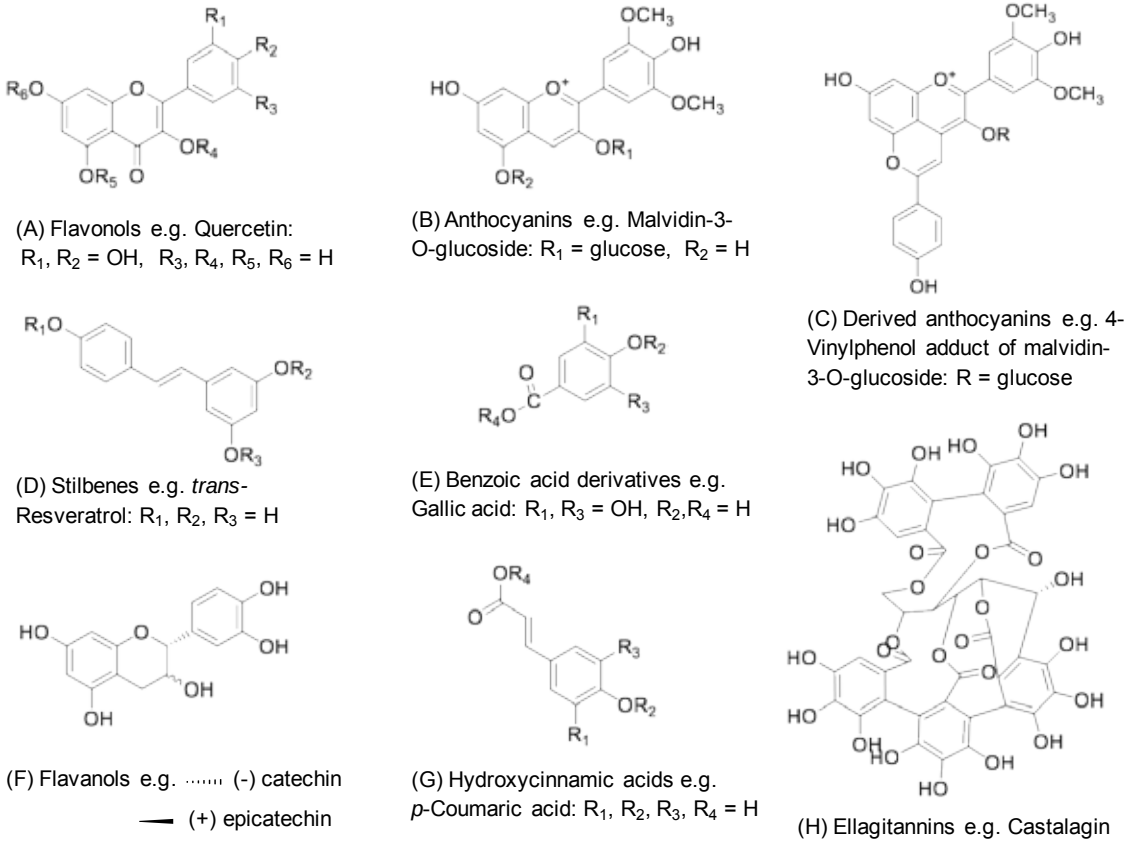


Figure 3.1. Structures of some representative examples of phenolic compounds: (A) flavonols, (B) anthocyanins, (C) derived anthocyanins, (D) stilbenes, (E) benzoic acid derivatives, (F) flavanols, (G) hydroxycinnamic acids and (H) castalagin.

LC coupled to ultraviolet–visible (UV/Vis) spectrophotometric detection (LC-DAD) offers an affordable and robust technique for the determination of phenolic compounds. Liquid chromatography coupled to DAD is inherently suited to provide information on the colour of these compounds and can also tentatively distinguish between the main phenolic structures since these display unique UV/Vis absorption spectra. Moreover, anthocyanins in particular may be detected with good sensitivity and selectivity at or near 520 nm, utilising an acidic mobile phase in reversed phase (RP) mode. However, LC-MS presents the most effective analytical tool for the study of phenolic compounds as it offers higher sensitivity, selectivity and specificity compared to LC-DAD, and it also yields structural information [5,6]. Furthermore, MS detection offers the distinct advantage of resolving peaks that co-elute in the chromatographic dimension, provided that the molecular masses (or fragment ions in tandem mass spectrometric

experiments) differ sufficiently. This is a very important consideration in the analysis of the highly complex families of phenolic compounds present in wines [7].

Phenolic compounds are amenable to various LC-MS atmospheric pressure ionisation techniques such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), employing ionisation in the positive (+) or negative (–) mode. In general, acidic mobile phases are preferred in chromatographic separations (for optimal efficiency), and low pH mobile phases generally favour positive ionisation. However, negative ionisation presents the additional advantage of detecting phenolic acids such as gallic acid, which plays an important role in the chemistry of wine phenolics [7,8].

Colour evolution of wine during ageing is a very complex process and LC-MS/MS (together with LC-UV/Vis and nuclear magnetic resonance spectroscopy (NMR)) has played an important role in the identification of the pigments. The oxidation of white wine is a well-known spoilage phenomenon that causes the development of brown colours and negative aromas and taste [9]. Phenolic compounds are good substrates for oxidation reactions and are therefore known precursors for browning reactions in white wines. Glutathione plays an important role in the prevention of enzymatic browning reactions through reactions with polyphenols such as hydroxycinnamates. Liquid chromatography – mass spectrometry provides a powerful tool for the study of these phenomena through identification of the resulting hydroxycinnamic acid derivatives present in white wines [10].

The combination of LC-ESI-MS/MS (+ and – ionisation) data and LC-DAD spectra has enabled the identification of new derivatives that enhanced the understanding of the extent of the involvement of glutathione in browning inhibition in white wines [10]. In white wines, flavanols such as (+)-catechin and (–)-epicatechin may be oxidised to yield yellow xanthylium cation pigments. Since sulphur dioxide is not efficient in preventing the oxidation of phenolic compounds in the presence of dissolved oxygen, ascorbic acid is often used as complementary antioxidant to react directly with molecular oxygen. Erythorbic acid, the diastereoisomer of ascorbic acid, is also a permissible antioxidant in many countries, and is more effective than ascorbic acid to prevent the production of red tints or pinking in white wine. LC-ESI(+)-MS has been used in combination with ultra pressure liquid chromatography – photo diode array detection (UPLC-PDA) and spectrophotometry to study the role of these antioxidants in reactions with (+)-catechin and (–)-epicatechin in model solutions. Xanthylium cation pigments

were found to be the major contributors to colour development, with (+)-catechin providing less yellow colouration for a given antioxidant. Erythorbic acid proved to be more efficient in preventing brown oxidative colouration in model solutions [9].

Liquid chromatography – mass spectrometry has been used extensively to study anthocyanin-derived pigments in red wines. In combination with LC-DAD and NMR, LC-MS, LC-MS/MS and liquid chromatography – quadrupole-time-of-flight mass spectrometry (LC-QTOF) has been used for the detection and characterisation of new pigments directly in wine. Wine constituents possessing a polarisable double bond, such as pyruvic acid, acetaldehyde or vinylphenol may undergo cyclo-addition reactions with anthocyanins to add a pyran ring to the anthocyanidin base. These pyranoanthocyanins impart changes in wine colour towards orange hues [11,12]. The presence of pyranoanthocyanin-vinylphenol pigments in aged red wine suggests a family comprising several compounds with great structural diversity. Although anthocyanins constitute the major precursors for the formation of new pigments in young red wines, their pyruvic acid derivatives may be even more important in later stages of colour evolution [13]. New families of anthocyanin-derived pigments have been found in aged red Port and lees, corresponding to a double pyranoanthocyanin arrangement linked by a methyne bridge. At acidic pH, these compounds display a turquoise blue hue [14]. Two new yellow pigments (at low pH) with structures corresponding to methyl-linked pyranomalvidin-3-glucoside and the respective coumaroyl derivative have also been found in aged red Port. These compounds may contribute to the orange-red colour of aged red wines [4].

Alcalde-Eon *et al.* used HPLC-DAD-ESI(+)-MS to study the qualitative and quantitative changes that occur in the anthocyanins and derived pigments during ageing of red wines [15]. Liquid chromatography with UV detection, LC-ESI(–)-MS^n and high resolution mass spectrometry (LC-ESI(–)-HR-MS) were used to study the effect of sulphur dioxide on condensation reactions involving flavanols and oak wood aldehydes in model solutions. These condensation products are known to affect red wine colour and astringency development. Sulphur dioxide was found to retard the rate of condensation reactions through preferential reactions with aldehyde moieties. Identification of some of the condensation products was achieved by a combination of interpreting MS/MS spectra and accurate mass determinations [16]. Hydroxycinnamic acid-tartaric acid esters were also identified and quantified in red wines using LC-ESI(–)-MS/MS and LC-ESI(–)-QTOF [8]. The effect of micro-oxygenation and oak barrel ageing on colour development in young wines was studied by analysing anthocyanin and anthocyanin-derived

compounds utilising LC-ESI(+)-MS and LC-DAD [17]. The utility of direct infusion positive ionisation atmospheric pressure photo-ionisation (APPI) QTOF and desorption ESI(+)-MS has also been demonstrated for the characterisation of anthocyanins in wine [18,19].

Model solutions are often used to study the reactions of wine phenolic compounds. Liquid chromatography coupled to DAD, LC-MS and NMR spectroscopy have been used to elucidate the pigments formed from a reaction involving (+)-catechin in model solution to first produce colourless dimeric reaction products, followed by the formation of various xanthylium pigments. These compounds have been successfully detected in red wine samples by RP-LC-ESI-MS [20,21,22].

The stilbene, *trans*-resveratrol has diverse beneficial physiological effects on mammals, of which cardio-protective, anticancer, antioxidant, antibacterial and anti-inflammatory properties are the most important. It is also a preventative agent of neurodegenerative processes such as Alzheimer's and Parkinson's diseases [23,24]. The resveratrol *cis*-isomer, and oligomeric stilbenes, appears to have lower biological activity [25]. Red wines are an important source of *trans*-resveratrol and its derivatives and analogues. For its analytical determination, HPLC methods coupled to DAD, electrochemical detection, fluorometric detection (FL) and MS have been described [25]. Direct injection LC-ESI(–)-MS/MS has been reported for the quantitation of *trans*-resveratrol. Better sensitivity for this compound was obtained, compared with LC-DAD and LC coupled to fluorescence detection (LC-FL) [26]. A combination of LC-DAD and LC-ESI-MS/MS (positive and negative ionisation) was used to characterise the methanol-extractable polyphenols of the stems of selected grapevine varieties. The main groups of polyphenols from this source comprise *trans*-resveratrol and catechin and their derivatives. The total stilbenoid content was found to be cultivar dependent [24]. The antiradical activities of resveratrol and its oligomers have been studied by investigating their quenching mechanism on $^1O_2$ using LC-ESI(–)-MS/MS and high resolution Fourier transform ion cyclotron resonance mass spectrometry (HR-FTICR-MS) [27]. A study reporting a quantitative method for the determination of the isomers of resveratrol used ESI in negative ionisation mode, as it produced better sensitivity compared to positive ionisation (which suffered from adduct formation effects) [28]. The complexity of the wine matrix frequently requires sample pre-treatment to selectively remove interferences; for example, the application of multi-walled carbon nano-tubes as on-line solid phase extraction (SPE) in fully automated, high throughput analysis of resveratrol isomers in wines with UPLC-ESI(–)-MS/MS [25]. Turbulent-flow chromatography (TFC) was also used

as on-line sample clean-up and pre-concentration technique with LC-APCI-MS to study flavonoids and resveratrol in wines. In this application, APCI was preferred over ESI as it produced good sensitivity without adduct formation, while APCI in negative ionisation produced better sensitivity compared to positive ionisation [23].

Phenolic compounds may also be used to differentiate wines according to geographical origin, variety and vintage. Jaitz *et al.* quantified 11 of the major (poly)phenols from different classes known to occur in red wines using LC-ESI-MS/MS in negative ionisation [3]. Structural isomers of catechin/epicatechin, *cis-/trans*-resveratrol and *cis-/trans*-para-coumaric acid were separated on a sub 2 µm particle RP column, allowing the determination of 11 phenolic compounds in 10 minutes. The profile of phenolic compounds and isomeric ratios were used for classification of wines, employing canonical discriminant analysis. The inclusion of the abovementioned isomeric pairs led to a substantial increase in the statistical significance of the results [3].

The concentrations and taste contribution of oak-derived ellagitannins and their transformation products in red wine were investigated utilising LC-MS/MS in multiple reaction monitoring (MRM) mode. These taste-active, non-volatile wine components contribute astringency and bitterness to red wines. A sensitive and robust method utilising direct injection of wine samples on RP-LC was developed using negative ionisation ESI. This technique offers sensitivity and selectivity, but requires matrix-matched calibration since the co-eluting wine matrix affects analyte ionisation. Castalagin was found to be the predominant ellagitannin in oak-matured wines, with concentrations in the parts per million (ppm) range [29]. Lignins and hydrolysable tannins are the principle flavour compounds released from oak heartwoods that are commonly used in ageing and maturation of wines and spirits. Triterpenes, which contribute bitterness and astringency, may also be extracted from oak, aided by the ethanol content of these products. Arramon *et al.* reported a highly sensitive LC-MS method, using single ion reaction (SIR) mode, for the quantitation of four triterpenes in oak heartwoods, wines and spirits [30]. The presence of two acidic groups on these compounds and their high molecular weights and polarity favour the use of negative ionisation ESI. Quantitation was with a combination of an internal standard and standard addition, while the method yielded limits of detection (LODs) in the low ppm range. Sample preparation consisted of consecutive extractions with diethyl ether and ethyl acetate to yield triterpene aglycones and glycosylated triterpenes, respectively. Because of the higher alcohol content and longer maturation time, these terpenes are extracted more efficiently into brandies compared to white and red wines. Although a high degree of variability was observed

for all the products investigated, these terpenes are expected to play an important role in the flavour of these beverages [30].

The sensitivity and selectivity inherent to LC-MS/MS has also been exploited in the analysis of archaeological artefacts to find evidence for early winemaking. Archaeological residues subjected to alkaline fusion yielded syringic acid, which is released from malvidin-3-glucoside, the main anthocyanin in red wines. Tartaric acid, rarely found at high concentrations in nature in sources other than grapes, also serves as a marker for wine in dry contexts (i.e. desert conditions). These markers have been determined with great sensitivity and selectivity using LC-MS/MS in negative ionisation [31-34].

3.3. Mycotoxins

Mycotoxins are small (molecular weight (MW) <700) toxic compounds produced as secondary metabolites by approximately 200 identified fungal species that may colonise crops and contaminate them in the field or after harvest [35,36]. Crops that are stored for more than a few days become a potential target for fungal growth and mycotoxin contamination, although toxin production can generally not be predicted with certainty [35-37]. Post-harvest fungal activity generally depends on the moisture content, humidity and storage temperature. During storage fungi tend to develop in isolated pockets. This places very high importance on sampling protocols to ensure representative samples for solid agricultural commodities [35,38,39]. More homogeneous samples may reasonably be expected for wines where the contaminants are in solution. Mycotoxins are highly nephrotoxic, neurotoxic, carcinogenic, immunosuppressive and estrogenic compounds, implicated as causative agents in human hepatic and extrahepatic carcinogenesis [36,40]. Aflatoxins and ochratoxins are mycotoxins of major significance and are generally produced post-harvest [35]. Due to the numerous species of fungi responsible for their production, mycotoxins comprise a structurally diverse group of compounds, with about 100 different species identified to date [36,41]. The structural diversity of these compounds generally necessitates diverse extraction and analytical methods, although the introduction of LC-MS based methodologies facilitates multi-toxin methods suitable for a range of structurally diverse toxins in a single chromatographic run. The utility of such multi-toxin methods stems from the fact that a single fungal species can produce different toxins and that a single agricultural commodity can be contaminated with different toxins due to the co-occurrence of various fungi [35,36,41]. A reliable risk assessment and monitoring strategy for agricultural commodities, including wine, requires rapid and efficient analytical methods for unambiguous identification

and accurate quantitation of mycotoxins. Liquid chromatography – mass spectrometry, together with gas chromatography mass spectrometry (GC-MS), gas chromatography electron capture detection (GC-ECD) as well as LC-FL and LC-DAD detection are used in the field of mycotoxin analysis. The complex matrix of agricultural commodities and low levels of occurrence of the toxins frequently require sample pre-treatment and a wide variety of protocols has been described for this purpose [35,36].

The ochratoxins consist of three congeners designated A, B and C, and are produced by several *Aspergillus* and *Penicilium* species. Ochratoxin A is the most significant of these as it is distinctly more toxic and prevalent than the other congeners. Ochratoxin A occurs in a variety of agricultural commodities, including wine [36]. The occurrence of ochratoxin A in wine is mostly the result of the use of contaminated grapes [35]. Ingestion of ochratoxin A in humans is mostly linked with food consumption (principally cereals), whereas wine is recognised as the second major source of intake (a presumed contribution of ~10-15% of total intake) [38,42]. Ochratoxin A contamination is also more frequent in red wines compared to rosé and white wines – a phenomenon ascribed to longer maceration periods used in the preparation of red wines [38,43,44]. However, climatic conditions, principally factors such as humidity and temperature which promote fungal growth, also play an important role [36,44-46]. The European Commission suggests a maximum level for ochratoxin A in wine of 2 µg/kg [47]. Analytical determination of ochratoxin A in wine is most frequently carried out using RP-LC coupled to FL or MS detection [38,42,44,45,48]. The carboxylic acid group present in the structure of ochratoxin A requires an acidic mobile phase for optimal chromatographic efficiency [49]. The low levels of occurrence of ochratoxin A in wine generally necessitate sample pre-concentration, most often using immunoaffinity columns [42]. More cost-effective sample clean-up and pre-concentration strategies involve LLE, RP-SPE as well as automated on-line SPE protocols [38,44,46]. The cost-effective use of solid-phase micro-extraction (SPME) in combination with LC-FL has also been described, although this method suffers from relatively poor sensitivity [50]. Immunoaffinity clean-up in combination with RP-LC-FL is widely used for determination of ochratoxin A in wine as it offers cost-effectiveness and simplicity, while producing very good sensitivity. The procedure for sample clean-up using immunoaffinity columns also uses no toxic solvents, as is the case with some LLE methods [42]. Mass spectrometric detection offers high specificity and sensitivity, but ESI suffers from matrix interference effects, necessitating the use of a suitable internal standard for quantitation [44,46]. On-line SPE coupled to LC-MS has additional advantages, such as high precision and sample throughput [46]. Bacaloni *et al.* also found that

negative ionisation ESI is more efficient than positive mode and that the intensity of the deprotonated ion signal may be enhanced with increased acidification. This phenomenon was ascribed to enhanced droplet formation due to increased ion reduction on the capillary surface in an acidic mobile phase, thus enabling the spray to effectively carry and transfer a negative charge excess to the analyte. Atmospheric pressure chemical ionisation (APCI) has been found to produce distinctly lower sensitivity for ochratoxin A analysis on account of extensive in-source fragmentation of the parent ion [36]. A comparison of triple quadrupole and hybrid quadrupole – ion trap MS detectors revealed that the former is more sensitive (by a factor of ~3.5) and produces two intense fragments for qualitative purposes. The hybrid quadrupole – ion trap design, with the third quadrupole operated as a linear ion trap with axial ion ejection capabilities, provides product ion scanning capabilities for enhanced analyte identification. The limits of quantitation (LOQs) obtained with two instruments were 0.01 ng/mL and 0.03 ng/mL, respectively [46].

It was recently reported that in addition to ochratoxin A, some strains of *Aspergillus niger* may also produce the mycotoxin fumonisin B_2. Since this pathogen may also be associated with grapes, where it causes bunch and/or berry rot, derived products such as grape juice and wine may become contaminated [51,52]. Fumonisin B_2 has been determined in wine using RP-LC-MS with positive ionisation ESI. Sample clean-up and pre-concentration was achieved with mixed mode reversed phase / cation exchange SPE or using immunoaffinity columns, while an isotopically labelled internal standard was used for quantitation [52]. A RP SPE protocol has also been described for sample pre-treatment in the determination of fumonisin B_2 in wine [51]. As is the case with ochratoxin A, the prevalence of fumonisin B_2 was found to be greater in red wines compared to white wines [52]. The LODs obtained using these methods were in the range 0.25-0.5 µg/L, while contaminated wines were found to contain fumonisin B_2 in the range 0.4-25 µg/L [51,52].

3.4. Amines

Biogenic amines are physiologically active amines, of which histamine and putrescine are the most important congeners found in wine [2,53]. *Oenococcus oeni* (formerly *Leuconostoc oenos*), the major bacterium inducing malolactic fermentation, is reported to be the primary source of histamine production in wine [2]. This process may even continue after the bacteria have died, as the enzyme remains active longer than the corresponding bacteria. Amines may also be produced *via* decarboxylation of amino acids by some spoilage bacteria, most notably

pediococci [2]. In wine, several amino acids may be decarboxylated to yield, in addition to histamine, tyramine and putrescine [54]. Some biogenic amines can induce blood-vessel constriction, headaches, hypertension and allergic reactions, although their concentrations in wine generally are insufficient to produce these physiological effects in humans [2]. Biogenic amines are mainly determined in wine using LC. Pre- or post-column derivatisation is required for fluorescence or UV/Vis absorption detection [53,54]. Sample clean-up and pre-concentration using LLE or SPE are routinely applied to improve sensitivity and selectivity with these methods [53]. Liquid chromatography coupled to electrospray ion trap mass spectrometry (LC-ESI-ITMS) has been described for the simultaneous determination of eight important biogenic amines in wine in a single chromatographic run without any sample pre-treatment. The LC-ESI-ITMS procedure was rapid, sensitive (LODs 0.47-40.1 µg/L) and specific, but required the use of an internal standard (heptylamine) for quantitative purposes as ESI suffers from suppression effects caused by the co-eluting wine matrix. In positive ionisation ESI, biogenic amines produce intense protonated molecular ion signals and generally produce as the base peak ions corresponding to the loss of ammonia. The acquisition of product ion spectra in full-scan mode enables highly specific compound identification. Eight biogenic amines were confirmed in wine samples, the most important of which were histamine (0.40-8.22 mg/L), putrescine (0.06-13.00 mg/L) and tyramine (0.03-3.20 mg/L) [54]. Biogenic amine analysis has also been described using LC-APCI(+)-MS with pre-column derivatisation utilising 1,2-naphthoquinone-4-sulphonate. No matrix suppression effects were observed with this mode of ionisation in the wine matrix. Derivatisation offers advantages such as improved chromatographic efficiency in RP mode and increased sensitivity through elution in an effluent that is better suited for desolvation and analyte introduction into the MS. The LODs for seven biogenic amines ranged from 30.8 to 441 µg/L, which is suitable for the determination of wine biogenic amines at natural concentrations. Putrescine was found to be the most abundant congener in eight wine samples (5-45 mg/L), while histamine (2-16 mg/L) and tyramine (2-9 mg/L) were also present in notable concentrations [53].

Heterocyclic aromatic amines are mutagenic, carcinogenic substances identified in foods, pyrolysis products of amino acids and proteins, as well as in beer and wine. These compounds have been determined in wine samples utilising HPLC-ESI(+)-MS/MS after sample clean-up consisting of continuous LLE with dichloromethane followed by SPE on anion-exchange columns and subsequent evaporative concentration. The analytes were grouped according to polarity and two deuterated internal standards were included to normalise extraction efficiency

for polar as well as apolar heterocyclic aromatic amines, while a third internal standard was used to normalise ionisation efficiency. The limits of quantitation were 0.5-7.5 ng/L for 14 heterocyclic aromatic amines. Red wines generally contained higher levels of these compounds compared to white wines and concentrations in the low ng/L range of some congeners were found [55].

3.5. Pesticide residues

Synthetic organic pesticides are used for disease, pest and weed control in agriculture. These compounds play a very important role in crop protection in modern viticulture, as exemplified by the fact that in Italy, the largest producer of grapes and wine in the world, more than 200 pesticides are registered for use in the vineyard [2,56]. Methods for pesticide analysis are therefore indispensable to ensure that grapes and wines are safe for human consumption. Liquid chromatography – mass spectrometry is suited for the recent trend towards multi-residue pesticide methods that exhibit higher sensitivity, selectivity and specificity. Multi-class pesticide residue methods for wine generally require sample pre-treatment such as LLE [57], SPE [57,58], SPME [59], hollow-fibre liquid-phase extraction [60] and recently also the quick, easy, cheap, effective, rugged and safe (QuEChERS) method [61]. Among these approaches, SPE offers a good compromise between robustness, rapidity, efficiency, potential for automation and solvent consumption for routine work in combination with LC-MS [56]. Economou *et al.* [56] developed a mixed mode RP-SPE method for use with LC-MS utilising positive ionisation ESI for the determination of 46 pesticides and their transformation products. Ionisation suppression caused by wine matrix components was found to be related to the level of dilution of the extracts and was more pronounced in the case of red wines compared to white wines. This phenomenon necessitated the use of matrix matched calibration solutions to ensure accurate quantitation. The method yielded LODs in the order of 0.01 mg/L for these multi-class pesticides and was therefore fully compliant with current European Union (EU) legislation.

N-Methyl carbamate pesticides are widely determined by post-column reaction LC-FL. Goto *et al.* [62] developed a fast LC-ESI(+)-MS method for direct analysis of *N*-methyl carbamate pesticides in wine samples. The method involved sample pre-treatment consisting of dilution and filtration only, and produced short analytical run times by utilising a short analytical column. Ionisation suppression effects necessitated the use of three separate isotopically labelled internal standards for quantitation of nine carbamate pesticides. Limits of detection in the order of 0.005 mg/L were achieved.

3.6. Aroma and taste components

Although wine aroma compounds have mostly been analysed by GC techniques, the use of LC-MS is advantageous in some applications. For example, 3-alkyl-2-methoxypyrazines, important aroma compounds in especially Sauvignon blanc wines, have been determined with great sensitivity using LC-MS/MS. This method used distillation and liquid extraction to produce highly concentrated extracts, which were analysed utilising the sample loading capacity, sensitivity and selectivity of LC-MS/MS to yield LODs of 0.03 ng/L for three methoxypyrazines [63].

N-Glucosyl ethanolamine is a taste-modulating flavour ingredient of wine and the presence of this compound was investigated in German Beerenauslese wines utilising different LC-MS methods. Since Beerenauslese wines may contain up to 10% sugar, preparative HPLC was used to achieve sample clean-up and pre-concentration by a factor of 20. Evaluation of ionisation techniques revealed that ESI in negative ionisation was more efficient (by a factor of approximately 10) compared to positive ionisation when a chloride atom was attached to the structure of the molecule *via* post-column addition of chloroform. An ion-trap instrument was used to quantify the target compound and levels of 1.1 and 4.0 µg/L were found in two wines. The masses of three characteristic MS^2 fragments, obtained utilising a triple quadrupole instrument, were used to unambiguously identify *N*-glucosyl ethanolamine [64].

Although volatile thiols generally exhibit unpleasant odours, 4-methyl-4-mercaptopentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) have been identified as qualitative contributors to the typical varietal aroma of some wines and are recognised as key aroma compounds in wine. The most abundant thiol is 3MH, with concentrations ranging from 100-3 500 ng/L. It has been hypothesized that the major biogenesis pathway for the production of 3MH is the conversion of S-3-(hexan-1-ol)-glutathione (G3MH) during alcoholic fermentation. Roland *et al.* identified and quantified G3MH in musts and confirmed the direct conversion to 3MH using nano-LC-ESI(+)-MS/MS. Sample preparation consisted of cation exchange and RP-SPE. Data were acquired in single reaction monitoring (SRM) mode, yielding quantitative as well as qualitative information using stable isotope dilution calibration. This work contributed new elements of understanding to the biogenesis pathway for the production of 3MH [65].

Some volatile phenols have also been associated with off-flavours of wines, such as 4-ethylphenol and 4-ethylguaiacol, produced by *Brettanomyces dekkera*. When the combined

concentration of these compounds exceeds 620 µg/L, this off-odour becomes too pronounced for the wine to be acceptable, while below 400 µg/L it may contribute favourably to the complexity of wine. These compounds have been determined in red and white wines using GC techniques as well as LC-MS/MS and HPLC-DAD-FL. For LC-MS/MS analysis, RP separation and ESI in negative ionisation was used and data were acquired in MRM mode. Wines were diluted with methanol and injected directly for LC-MS/MS analysis. Quantitation was achieved using external standards, and the method produced an LOD of 10 µg/L. Simultaneously, qualitative confirmation was obtained by acquiring multiple product ions. For HPLC-DAD-FL analysis, direct injection and reversed phase gradient separation was employed with detection of the analytes at 280 nm (DAD), and 260 nm (excitation) and 305 nm (emission) respectively, for FL detection. Calibration was performed by standard addition, as matrix interferences were present in this analysis. The LODs of the method were 10 µg/L and 1 µg/L, respectively, for DAD and FL detection. These methods were suitable for quantitative and qualitative determination of 4-ethylphenol and 4-ethylguaiacol in wines affected by microbial contamination with yeasts of the *Brettanomyces* genus [66].

Taste and mouth-feel are very important wine quality parameters. The non-volatile taste-active compounds of five different Tempranillo wines were investigated by semi-preparative HPLC fractionation and subsequent sensory analysis of the fractions [67]. Bitter and particularly astringent compounds were quantified in these fractions using a dedicated UPLC-MS method. The results showed that wine bitterness and astringency cannot readily be related to these properties of the fractions, and therefore must be the result of perceptual and physicochemical interactions. Bitter character was attributed to some flavonols. Astringency was not due to proanthocyanidin monomers, dimers, trimers or tetramers (galloylated or non-galloylated). The most important compounds producing astringency were *cis*-aconitic acid, followed by vanillic and syringic acids, as well as ethyl syringate [67].

Varietal characterisation of non-aromatic Falanghina grapes and wines has been achieved through fingerprinting volatile compounds and their precursors. A combination of GC-MS, LC-ESI-MS and MALDI-TOF-MS techniques, together with specific methodologies for sample extraction and purification, were used to determine terpenes, terpene glycosides and norisoprenoids in these products. Specific markers were identified for authentication of varietal and origin claims under the relevant European Appelation of Origin designations [68].

3.7. Metals

Arsenic is present in soil, water, air and all living organisms. Due to industrial activity, its concentration in the environment is increasing. In wine, the presence of arsenic depends on the soil type, but may also result from application of herbicides, insecticides, and production and storage conditions. The maximum total arsenic concentration in wine has been set to 10 µg/L by the World Health Organisation. Different arsenic species exhibit differing toxicities. For example organo-arsenic compounds such as arsenobetaine are relatively harmless, whereas inorganic arsenic species such as arsenite are more toxic. Liquid chromatography coupled to inductively coupled plasma – mass spectrometry (LC-ICP-MS) provides an ideal tool for arsenic speciation since different arsenic compounds can be separated in the chromatographic dimension (isocratic elution in anion-exchange mode), while ICP-MS provides very sensitive and selective detection of the separated species [69]. Quantitation of arsenic species by LC-ICP-MS has been performed with external standard calibration, yielding LODs ranging from 0.10 to 0.21 µg/L. Arsenic (V) is the most abundant species found in wines [69].

3.8. Conclusions

In this chapter, various applications that demonstrate the increasing use and importance of LC-MS in wine analysis have been described. For example, 3-alkyl-2-methoxypyrazines, which have generally been determined with GC exclusively, have recently been analysed, with high sensitivity using LC-MS. Liquid chromatography – mass spectrometry has also been used extensively for analysis and structure elucidation studies of wine polyphenols and related derivatives. Electrospray ionisation is the most widely used ionisation technique, but suffers matrix effects. Atmospheric pressure chemical ionisation is a very robust alternative to ESI, but is generally less sensitive than ESI. Liquid chromatography – tandem mass spectrometry is principally used for very sensitive and specific targeted analysis. Liquid chromatography – mass spectrometry in scan mode, as well as LC-MS/MS and LC-QTOF, are used as structural elucidation tools to unravel wine chemistry.

References

[1] M.C. McMaster, LC/MS: A practical user's guide, John Wiley & Sons Inc., New Jersey, U.S.A. (2005).
[2] R.S. Jackson, Wine science: principles, practice and perception, Academic Press, San Diego, U.S.A. (2000).
[3] L. Jaitz, K. Siegl, R. Eder, G. Rak, L. Abranko, G. Koellensperger, S. Hann, Food Chem. 122 (2010) 366.
[4] J. He, C. Santos-Buelga, A.M.S. Silva, N. Mateus, V. De Freitas, J. Agric. Food Chem. 54 (2006) 9598.
[5] B. Abad-García, L.A. Berrueta, S. Garmón-Lobato, B. Gallo, F. Vicente, J. Chromatogr. A 1216 (2009) 5398.
[6] B. Berente, D. De la Calle García, M. Reichenbächer, K. Danzer, J. Chromatogr. A 871 (2000) 95.
[7] S. Pérez-Magariño, I. Revilla, M.L. González-SanJosé, S. Beltrán, J. Chromatogr. A 847 (1999) 75.
[8] F. Buiarelli, F. Coccioli, M. Merolle, R. Jasionowska, A. Terracciano, Food Chem. 123 (2010) 827.
[9] A.C. Clark, J. Vestner, C. Barril, C. Maury, P.D. Prenzler, G.R. Scollary, J. Agric. Food Chem. 58 (2010) 1004.
[10] M.J. Cejudo-Basante, M.S. Pérez-Coello, I. Hermosín-Guitiérrez, J. Agric. Food Chem. 58 (2010) 11483.
[11] E.M. Francia-Aricha, M.T. Guarra, J.C. Rivas-Gonzalo, C. Santos-Buelga, J. Agric. Food Chem. 45 (1997) 2262.
[12] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutounet, Phytochem. 47 (1998) 1401.
[13] N. Mateus, J. Oliveira, J. Pissarra, A.M. González-Paramás, J.C. Rivas-Gonzalo, C. Santos-Buelga, A.M.S. Silva, V. De Freitas, Food Chem. 97 (2006) 689.
[14] J. Oliveira, J. Azevedo, A.M.S. Silva, N. Teixeira, L. Cruz, N. Mateus, V. De Freitas, J. Agric. Food Chem. 58 (2010) 5154.
[15] C. Alcalde-Eon, M.T. Escribano-Bailón, C. Santos-Buelga, J.C. Rivas-Conzalo, Anal. Chim. Acta 563 (2006) 238.
[16] M.F. Nonier, N. Vivas, N. Vivas de Gaulejec, C. Absalon, C. Vitry, Food Chem. 122 (2010) 488.
[17] M. Cano-López, J.M. López-Roca, F. Pardo-Minguez, E. Gómez Plaza, Food Chem. 119 (2010) 191.
[18] J.L. Gómez-Ariza, T. García-Barrera, F. Lorenzo, Anal. Chim. Acta 570 (2006) 101.
[19] L. Hartmanova, V. Ranc, B. Papouskova, P. Bednar, V. Havlicek, K. Lemr, J. Chromatogr. A 1217 (2010) 4223.
[20] N.E. Es-Safi, C. Le Guerneve, H. Fulcrand, V. Cheynier, M. Moutounet, Int. J. Food Sci. Techn. 35 (2000) 63.
[21] N.E. Es-Safi, C. Le Guerneve, V. Cheynier, M. Moutounet, Tetrahedron Lett. 41 (2000) 1917.
[22] N.E. Es-Safi, C. Le Guerneve, V. Cheynier, M. Moutounet, J. Agric. Food Chem. 48 (2000) 4233.
[23] M.A. Presta, B. Bruyneel, R. Zanella, J. Kool, J.G. Krabbe, H. Lingeman, Chromatographia Supplement 69 (2009) S167.
[24] T. Püssa, J. Floren, P. Kuldkepp, A. Raal, J. Agric. Food Chem. 54 (2006) 7488.
[25] Y. Lu, Q. Shen, Z. Dai, J. Agric. Food Chem. 59 (2011) 70.
[26] F. Buiarelli, F. Coccioli, R. Jasionowska, M. Merolle, A. Terracciano, Chromatographia 64 (2006) 475.
[27] L. Jiang, S. He, K. Jiang, C. Sun, Y. Pan, J. Agric. Food Chem. 58 (2010) 9020.

[28] M. Careri, C. Corradini, L. Elviri, I. Nicoletti, I. Zagnoni, J. Agric. Food Chem. 52 (2004) 6868.
[29] T. Stark, N. Wollmann, K. Wenker, S. Lösch, A. Glabasnia, T. Hofmann, J. Agric. Food Chem. 58 (2010) 6360.
[30] G. Arramon, C. Saucier, S. Tijou, Y. Glories, LCGC North America 21, 9 (2003) 910.
[31] H. Barnard, A.N. Dooley, G. Areshian, B. Gasparyan, K.F. Faull, J. Arch. Sci. Doi: 10.1016/j.jas.2010.11.012.
[32] M.R. Guasch-Jané, C. Andrés-Lacueva, O. Jáuregui, R.M. Lamuela-Raventós, J. Arch. Sci. 33 (2006) 1075.
[33] M.R. Guasch-Jané, C. Andrés-Lacueva, O. Jáuregui, R.M. Lamuela-Raventós, J. Arch. Sci. 33 (2006) 98.
[34] M.R. Guasch-Jané, M. Ibern-Gómez, C. Andrés-Lacueva, O. Jáuregui, R.M. Lamuela-Raventós, Anal. Chem. 76 (2004) 1672.
[35] N.W. Turner, S. Subrahmanyam, S.A. Piletsky, Anal. Chim. Acta 632 (2009) 168.
[36] P, Zöllner, B. Mayer-Helm, J. Chromatogr. A 1136 (2006) 123.
[37] D. Garcia, A.J. Ramos, V. Sanchis, S. Marín, Food Microbiol. 26 (2009) 757.
[38] I.K. Cigić, M. Strlič, A. Schreiber, M. Kocjančič, B. Pihlar, Anal. Lett. 39 (2006) 1475.
[39] J. Feizy, H.R. Beheshti, N. Khoshbakht Fahim, S.S. Fakoor Janati, G. Davari, Food Add. Cont. Part B 3 (2010) 263.
[40] T.E. Massey, R.K. Stewart, J.M. Daniels, L. Ling, Proc. Soc. Exp. Biol. Med. 208 (1995) 213.
[41] A.K. Malik, C. Blasco, Y. Picó, J. Chromatogr. A 1217 (2010) 4018.
[42] A. Visconti, M. Pascale, G. Centonze, J. Chromatogr. A 864 (1999) 89.
[43] P. Battilani, A. Pietri, Eur. J. Plant Pathol. 108 (2002) 639.
[44] A. Leitner, P. Zöllner, A. Paolillo, J. Stroka, A. Papadopoulou-Bouraoui, S. Jaborek, E. Anklam, W. Lindner, Anal. Chim. Acta 453 (2002) 33.
[45] C. Dall' Asta, G. Galaverna, A. Dossena, R. Marchelli, J. Chromatogr. A 1024 (2004) 275.
[46] A. Bacaloni, C. Cavaliere, A. Faberi, E. Pastorini, R. Samperi, A. Lagana, J. Agric. Food Chem. 53 (2005) 5518.
[47] Regulation (EC) No. 123/2005, Off. J. Eur. Communities L 25/3 (2005).
[48] C. Tessini, C. Mardones, D. von Baer, M. Vega, E. Herlitz, R. Saelzer, J. Silva, O. Torres, Anal. Chim. Acta 660 (2010) 119.
[49] M. Becker, P. Degelmann, M. Herderich, P. Schreier, H-U. Humpf, J. Chromatogr. A 818 (1998) 260.
[50] A. Aresta, R. Vatinno, F. Palmisano, C.G. Zambonin, J. Chromatogr. A 1115 (2006) 196.
[51] A. Logrieco, R. Ferracane, A. Visconti, A. Ritieni, Food Add. Cont. 27 (2010) 1136.
[52] J.M. Mogensen, T.O. Larsen, K.F. Nielsen, J. Agric. Food Chem. 58 (2010) 4853.
[53] N. García-Villar, S. Hernández-Cassou, J. Saurina, J. Chromatogr. A 1216 (2009) 6387.
[54] S. Millán, M.C. Sampedro, N. Unceta, M.A. Goicolea, R.J. Barrio, Anal. Chim. Acta 584 (2007) 145.
[55] E. Richling, C. Dekker, D. Häring, M. Herderich, P. Schreier, J. Chromatogr. A 791 (1997) 71.
[56] A. Economou, H. Botitsi, S. Antoniou, D. Tsipi, J. Chromatogr. A 1216 (2009) 5856.
[57] G.F. Pang, C.L. Fan, Y.M. Liu, Y.Z. Cao, J.J. Zhang, B.L. Fu, X.M. Li, Z.Y. Li, Y.P. Wu, Food Add. Cont. 23 (2006) 777.
[58] M.J. Nozal, J.L. Bernal, J.J. Jimenez, M.T. Martin, J.Bernal, J. Chromatogr. A 1076 (2005) 90.
[59] J. Wu, C. Tragas, H. Lord, J. Pawliszyn, J. Chromatogr. A 976 (2002) 357.
[60] P. Plaze Bolãnos, R. Romero-Gonzáles, A. Garrido Frenich, J.L. Martínez Vidal, J. Chromatogr. A 1208 (2008) 16.

[61] P. Payá, M. Anastassiades, D. Mack, I. Sigalova, B. Tasdelen, J. Oliva, A. Barba, Anal. Bioanal. Chem. 389 (2007) 1697.
[62] T. Goto, Y. Ito, H. Oka, I. Saito, H. Matsumoto, H. Sugiyama, C. Ohkubo, H. Nakazawa, H. Nagase, Anal. Chim. Acta 531 (2005) 79.
[63] P. Alberts, M.A. Stander, S.O. Paul, A. de Villiers, J. Agric. Food Chem. 57 (2009) 9347.
[64] E. De Rijke, N. Bouter, B.J. Ruisch, S. Haiber, T. König, J. Chromatogr. A 1156 (2007) 296.
[65] A. Roland, R. Schneider, C. Le Guernevé, A. Razungles, F. Cavelier, Food Chem. 121 (2010) 847.
[66] P. Caboni, G. Sarais, M. Cabras, A. Angioni, J. Agric. Food Chem. 55 (2007) 7288.
[67] M-P. Sáenz-Navajas, V. Ferreira, M. Dizy, P. Fernández-Zurbano, Anal. Chim. Acta. 673 (2010) 151.
[68] A. Nasi, P. Ferranti, S. Amato, L. Chianese, Food Chem. 110 (2008) 762.
[69] C.M. Moreira, F.A. Duarte, J. Lebherz, D. Pozebon, E.M.M. Flores, V.L. Dressler, Food Chem. 126 (2011) 1406.

CHAPTER 4

Analytical techniques for wine analysis: An African perspective *

* Published as A. de Villiers, P. Alberts, A.G.J. Tredoux, H.H. Nieuwoudt, Anal. Chim. Acta 730 (2012) 2-23.

4.1. Introduction

Mankind has been involved with winemaking since ancient times. Wine holds a special place in many countries and cultures and man could have encountered some of his earliest experiences in chemical reactions through the processes of fermentation and oxidation of wine. Historical records show the earliest winemaking activities in Mesopotamia and Caucasus by 6000 BC [1]. Colonisation by the Romans of regions around the Mediterranean Sea resulted in the spread of the cultivation of the vine plant. Earliest records of winemaking on the African continent trace activities to the southern shores of the Mediterranean as early as 5000 BC and confirm ancient Egypt as the first winemaking region in Africa [1]. Much has been written about wine and ancient Egyptian civilisation; historical records show that it was served to noblemen and pharaohs, and stored in individual jars clearly marked with details of winemaker, vintage and vineyard. From Egypt, cultivation of the vine spread to other northern African regions with all the vineyards being close to the coast. The vine *Vitis vinifera* was introduced to the southern tip of the African continent by European explorers in the seventeenth century [1]. In 1655 Dutch settlers planted French vine cuttings on the lower slopes of Table Mountain in the Cape of Good Hope, South Africa. As early as the eighteenth century, Vin de Constance wines from the area now known as Constantia were amongst the world's most sought after [2]. The early vineyard plantings accelerated with the settlement of French Huguenots in the Cape during the late 17th century and early 18th century.

The major African wine producing regions are those with Mediterranean climate, typically with mild winters and dry, hot summers, in which the vine thrives. On the African continent, only very small regions located in Northern Africa and in the southern tip of the continent, the coastal areas of the Western Cape, fit this description. Today, the Northern African countries Algeria, Morocco and Tunisia have established wine industries with important intra-African and African–European export components. Wine labelling laws are based on the French system of *Appelation d'Origine Controleé* and a strong influence of French wine grape cultivars like Cabernet Sauvignon, Syrah, Mourvedre, Carignan, Ugni Blanc and Clairette is seen in plantings [1]. Muscat wines, that can be sweet or dry, are especially successful in Tunisia. Algeria annually produces about 600,000 hectolitres of wine, and the wine provinces Oran and Alger are renowned for red wine, while smaller quantities of rosé and white wine are also produced. Morocco has 15,000 hectares planted under vineyards, of which about 85% produce red wine and the rest rosé and a pale white wine. Well-known Moroccan wine regions include Rabat, and the coastal vineyards of Casablanca, Meknes and Fez.

South Africa is the principal wine producing country in Southern Africa, with about 60 appellations within the Wine of Origin (WO) scheme and a tiered system of wine regions, districts and wards [2]. Annual production of more than 100 million bottles places the country as the world's 7th largest wine producer. The area covered by South African vine plantings constitutes 1.3% of the world's vineyards [3]. Renowned wine regions include Constantia, Stellenbosch, Franschhoek, Overberg and Robertson. Well-known white wine grape cultivars are Chenin blanc, Sauvignon blanc, Chardonnay, Muscat d'Alexandrie and Colombar, while red varieties include Cabernet Sauvignon, Shiraz, Merlot and Pinotage [2]. The South African wine industry is dependent on exports and the wine quality is comparable with the world's best.

As in all areas of food and beverage production, the analysis of wine plays an essential role in the industry. Accurate analytical measurements are required at all stages of the winemaking process, from the vineyard, the weighbridge where grapes are delivered, during the fermentation and maturation stages, during bottling and through to certification (Figure 4.1). These measurements are required for various reasons. Firstly, analytical methods are used to provide information required by law for the production and marketing of these products. This includes regulatory analysis pertaining to the marketing and sale of these products in an increasingly competitive international market, which therefore has important financial implications. Secondly, from a research and development perspective, analysis is also used to shed light on more fundamental aspects such as the microbiological, genetic, physiological and chemical processes involved in grape and wine production. While obviously important from a production perspective, this research also contributes to the fundamental understanding of the chemical composition of natural products in general and the production of commodities useful for human consumption from these products.

Analysis of wine related products involves the use of an extremely wide variety of analytical techniques, reflecting the equally diverse goals of these analyses. The range of methods used for wine analysis mirrors to some extent the varied information relevant to wine producers and researchers. Techniques used vary between relatively simple wet-chemical methods and highly complex (and expensive) instrumental methods capable of detailed investigation of individual chemical constituents. Generally, the former types of methods are used for routine analyses aimed at demonstrating compliance with product legislation, since these methods are relatively cheap and may be performed in many laboratories. On the other hand, there is an increasing international trend of applying more advanced instrumentation for high-level research of wine and derived products. The inherent inter-

disciplinary nature of analytical research in this field has contributed to improving the quality of grape-derived products as well as new scientific knowledge.

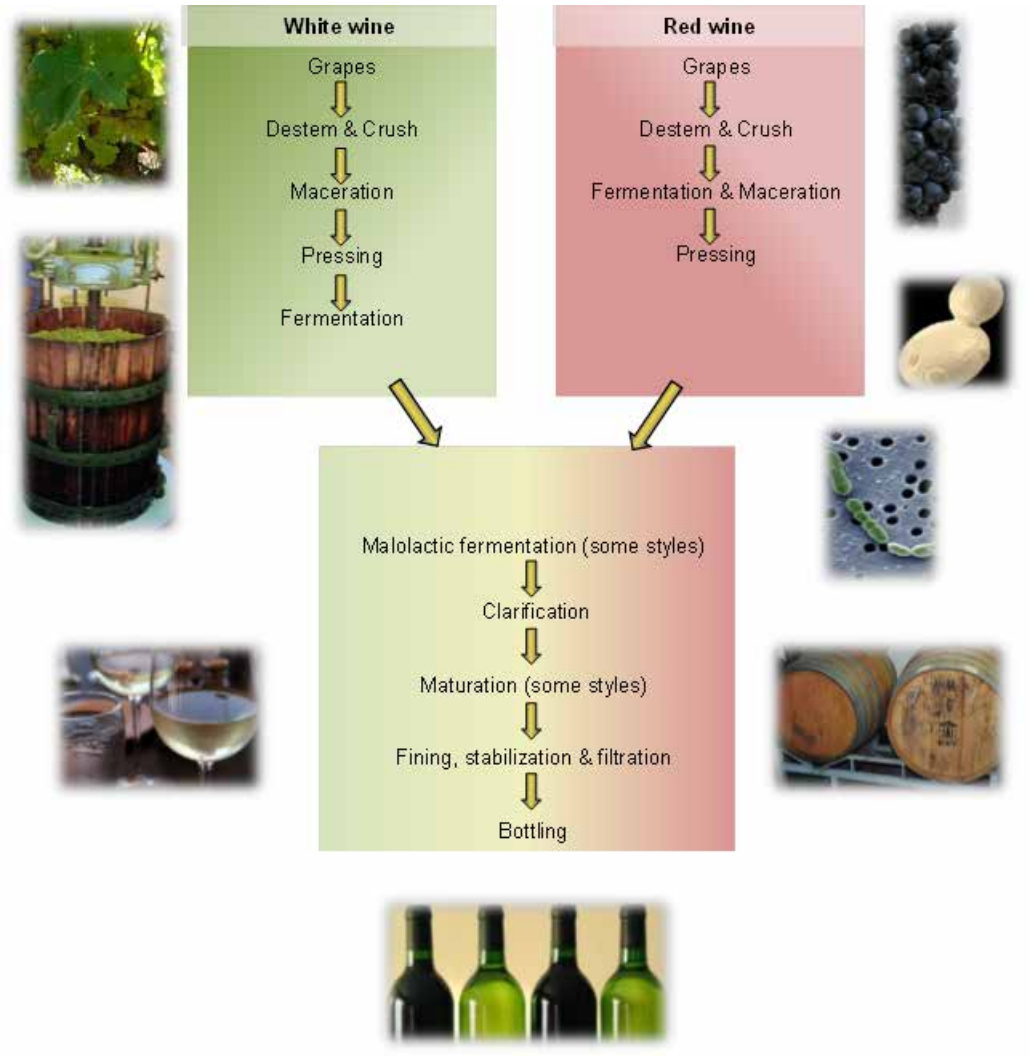


Figure 4.1. Schematic illustration of the different steps involved in the winemaking processes of red and white wines. Adapted from [63].

Analysis of grape-derived products on the African continent, to a large extent, reflects current international trends: ongoing development in analytical chemistry instrumentation and methods has resulted in the increased application of advanced spectroscopic and chromatographic methods. This review seeks to provide an overview of the analysis of wine, grapes, and their derived products as performed on the African continent. For the purposes of this review, literature reports including at least one author affiliated to an African institution are included. Furthermore, the focus is exclusively on the application of advanced instrumental analytical methods for grape and wine analysis. In the context used here, instrumental analytical techniques refer primarily to spectroscopic, chromatographic and

electrophoretic methods. Figure 4.2 provides a graphic summary of the most important instrumental analytical techniques used for analysis of grapes and wine in Africa.

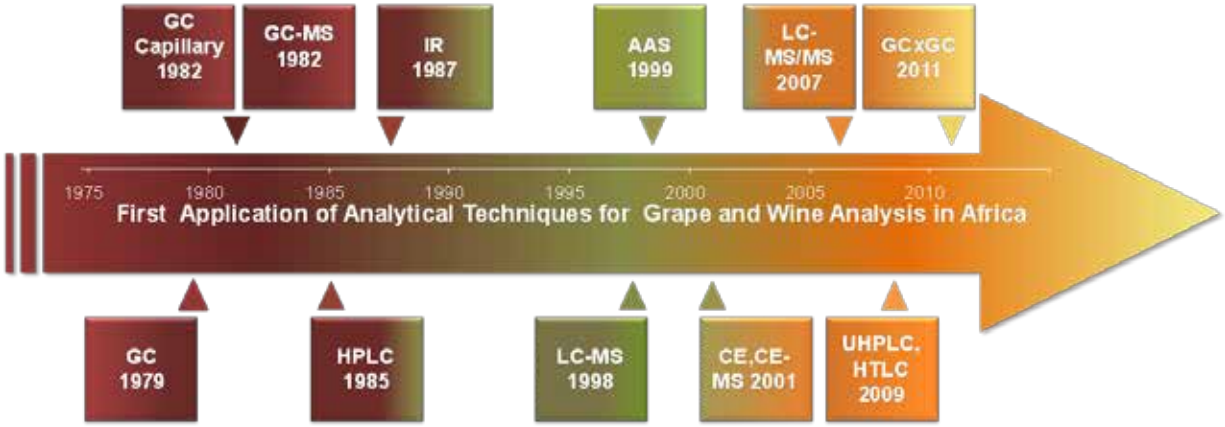


Figure 4.2. Summary of the most important developments in instrumental analytical techniques applied to wine analysis in Africa since 1975. Arrows indicate the first published report of a particular method for wine analysis by African scientists. The relevant references for each application are: GC [49], capillary GC [56], GC-MS [56], HPLC [100], IR [32], LC-MS [152], AAS [43], CE, CE-MS [164], LC-MS/MS [159], GC´ GC [79,80,83].

4.2. Spectroscopic analysis of wines: Global perspectives

Global production figures for 2008 recorded about 7800 million hectares under wine grapes and in excess of 240 million hectolitres of wine being produced [4]. These huge volumes make it clear that rapid, low-cost and environmentally friendly analytical methods are of critical importance to maintain sustainability of the international wine industry. This is particularly true on the African continent, where demands on existing natural resources, notably water and energy, are already high.

Spectroscopic methods applied for wine and grape analyses include a wide range of techniques, spanning atomic spectroscopic methods such as atomic absorption spectroscopy (AAS) [5] and inductively coupled plasma (ICP), and several molecular spectroscopic methods such as infrared (IR) and ultraviolet/visible (UV/VIS) spectrophotometry, nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). Some of these technologies are extensively used in international wine research, but have not yet been exploited in Africa, and hence will not be covered in this review. For example, NMR is widely used globally for wine analysis, notably for authentication purposes [6]. In addition, recent developments in near-infrared (NIR)

spectroscopy for remote sensing of vineyards [7] as well as development of portable devices for non-destructive monitoring of grape quality [8] have not yet found application in African wine research. Finally, while MS may be used directly for wine analysis [9], in the African content, it has been used exclusively in hyphenated chromatographic and spectroscopic systems.

Several features of spectroscopic techniques, particularly of UV/Vis spectrophotometry and IR spectrometry, offer attractive features that make them ideally suited for handling very large volumes of the essential routine grape and wine analyses [10]. Ultraviolet-visible spectrophotometric methods are used extensively for determination of colour and phenolic compounds in grapes and wine [11,12] – features that have shown to be important drivers of preference amongst consumers [13]. For example, absorbance measurements at 280 nm are used for the quantitation of total phenolics and at 520 nm for anthocyanins. Although the lack of specificity in these methods (compared to liquid chromatography) can result in overestimation of the phenolic content, spectrophotometric analysis nevertheless provides a rapid and inexpensive methodology particularly suited for high sample throughput [11,12]. Despite its utility, UV/Vis instrumentation has not seen much innovation in recent years.

Vibrational spectroscopy, both in the near- and mid-infrared regions, has recently received considerable attention in grape and wine analysis and the past two decades have seen a surge in quantitative and authentication applications in international wine industries [14-19]. Chemometrics is indispensable for interpretation of spectroscopic data and refers to a vast field of statistical and mathematical techniques that are used to extract relevant information from primary chemical or analytical measurements [20-22]. Typical problems addressed by spectroscopic data combined with chemometrics include multivariate calibration and classification [17,18,20], process monitoring [23,24], quality control and data display [21]. These applications address quantitative and qualitative challenges such as product authentication in grape and wine analysis. Recent improvements in instrument hardware combined with powerful chemometric software packages, which are nowadays integrated with instrument software, undoubtedly made a significant contribution to these developments.

4.2.1. Vibrational spectroscopy in wine analysis

Vibrational spectroscopy offers several advantages and much has been written about these [14,25]. The technology is non-destructive, and by nature of its indirect measurement, also reagentless, while no toxic waste is generated. Analysis time is in the seconds range and the technology can be fully automated, including processing and distribution of the analytical results. Very little sample preparation is required; mostly the only requirement is a filtration step to remove large particles from liquids and a degassing step for Fourier transform mid infrared (FT-MIR) analysis that is achieved by simple vacuum filtration or sonication [26,27]. Drawbacks of the technology are the relatively high initial instrumentation cost, as well as the intensive calibration procedures that are a prerequisite for implementation of the technology.

Vibrational spectroscopy is based on the measurement of the frequencies of the vibrations of covalent bonds in functional groups upon absorption of radiation in the near-infrared (NIR) and mid-infrared (MIR) regions [28]. The NIR region is usually defined as ranging from 800-2500 nm, while the MIR region from 4000-400 cm^{-1} (2500-2.5 × 10^4 nm). In instrumentation, the exact wavelength range of these regions is customised to suit specific applications, and the visible region is combined with the NIR range in some spectrometers. The main difference between the NIR and MIR regions is that absorption of MIR light by matter causes fundamental vibrations of covalent bonds, whereas absorption of NIR light results in overtones and combination bands [28]. The result is that MIR spectra show higher specificity than NIR spectra, and are therefore frequently preferred for quantitative applications. NIR light is not absorbed as well by matter as MIR light and is better suited for measuring whole fruits [29]. The measured frequencies in NIR and MIR spectra are processed through a series of mathematical procedures (which may include Fourier transformation) to calculate an absorbance spectrum. The latter, in turn, is correlated to the actual concentrations of the relevant components in the sample matrix through a calibration process that involves multivariate statistical procedures such as principal component analysis (PCA), principal component regression (PCR) and partial least squares (PLS) regression [20,30]. The application of FT-IR for the routine analysis of wine has recently received much attention [14,25].

The NIR spectrum of wine is dominated by two large absorption bands that correspond to O–H bonds around 1400 nm and 1900 nm, corresponding to water and ethanol, respectively [16]. The MIR spectrum is dominated by strong absorbance of water in the regions 1716 to 1543 cm^{-1} and 3626 to 2970 cm^{-1}. The region from 929 to 1600 cm^{-1} is referred to as the ‘fingerprint’ area, and is particularly useful in molecular absorption spectroscopy because

many different IR bands corresponding to the vibrations of the C–O, C–C, C–H and C–N bonds occur in this region [28]. The region from ~5000 cm^{-1} to 3626 cm^{-1} does not contain much useful information. This area, as well as both water absorption areas, is frequently excluded in multivariate data analysis, due to the noise introduced in the IR spectra from these regions [26]. The utility of chemometric techniques for the design of PLS calibration sets was demonstrated with the use of PCA to identify the main sources of variation in a set of 329 South African wines [26]. The set included wines belonging to various styles: noble late and special late harvest wines (sugar levels ranging from 31 to 147 g/L), wooded and unwooded dry red and white wines, off-dry white wines and young wines (sugar levels collectively ranging from 0.5 to 13 g/L). Principal component 1 (PC1) (that explained 96% of the variation) seemed to distinguish between samples based on sugar content (Figure 4.3), while PC2 differentiated between samples based on alcohol content. Principal Component Analysis (PCA) results clearly separated the different wine styles, illustrating the potential of FT-MIR spectroscopy to be used for style identification and verification.

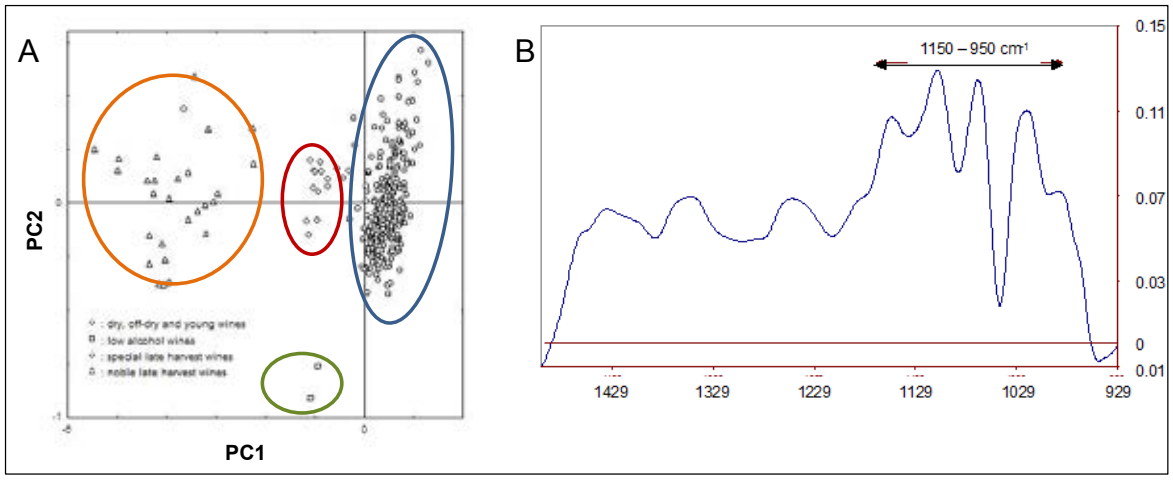


Figure 4.3. (A) PCA score plot, PC1 versus PC2, based on FT-MIR spectra of different wine styles: dry, off-dry and young wines (blue, circles); low alcohol wines (green, squares); special late harvest wines (red, diamonds); noble late harvest wines (orange, triangles). (B) PC1 loadings plot in the wavenumber region 1500-929 cm^{-1}. Reprinted with permission from [26].

The use of vibrational spectroscopy for quantitation of wine compounds was first reported for filter-based NIR instruments where only a small number of wavelengths were available for measurements [31]. One of the early applications for wine analysis on a filter-based NIR instrument was the quantitation of ethanol [38]. Contemporary NIR instrumentation includes, amongst others, acousto-optical tunable filter instruments (AOTF), photo diode array and

Fourier transform (FT-NIR) interferometer systems [33]. Hyphenated instruments such as UV/Vis or Vis-NIR have also been used in wine and grape analyses [16]. Nowadays, the focus has moved from NIR spectroscopy to MIR spectroscopy for the routine analysis of wine, due to more accurate determination of a wider range of compounds [34].

The marketing of Fourier transform mid-infrared (FT-MIR) instrumentation dedicated to routine wine analysis in 1998 (WineScan FT 120, Foss A/S, Denmark) provided a huge impetus to the implementation of infrared technology. The instrument is fitted with a Michelson interferometer and a 37 µm CaF_2 cuvette that is temperature controlled. Spectra are generated in transmission mode and sample volumes of ~30 mL are needed [35]. In terms of software, so-called 'global calibrations' for the quantitation of a wide range of wine compounds and properties are available, including levels of glucose, fructose, organic acids (tartaric acid, malic acid, acetic acid, lactic acid, gluconic acid, sorbic acid, citric acid), ethanol, density, CO_2, polyphenols, glycerol, pH, iron, copper, colour, ethanol, ethyl acetate and methanol. These parameters can be quantified in a single analysis for a wide range of wine styles and in the ranges normally found in grapes and wine [36]. Typical analysis time, including sample preparation, is less than one minute. Instrumentation with sample presentation modes in attenuated total reflection (ATR) have recently become available and have been used for routine analysis of wine [34]. A wide selection of materials is used for the sampling plates including diamond, Si, ZnSe and Ge. Advantages of FT-MIR ATR instruments include small sample volumes required (less than 0.2 mL), samples are placed directly onto the ATR platform, much smaller physical dimensions than conventional laboratory instrumentation, and lower cost, which makes it an attractive option for commercial laboratories [34]. Currently, analytical instruments suitable for multi-component analyses are available with impressive performance data in terms of accuracy, precision and speed of analysis.

Researchers at Stellenbosch University have focussed on the development of quantitative and qualitative applications using IR spectroscopy in viticulture and oenology. This collaborative research combines expertise in the application of chemometric methods, primarily from Europe, with the African partners' expertise in winemaking and viticulture. This culminated in the formation of the Chemometrics Society of South Africa [22] and the first African-European conference on chemometrics *Data modelling in Biological Sciences and Industrial Processing,* held in Rabat, Morocco in 2010 [37]. The long term ambition of this initiative is to strengthen ties between European and African countries in projects where chemometrics is the major focus areas.

IR spectroscopy has been applied to all stages of the wine production chain in South Africa, ranging from the vineyard to the bottled product. The utility of NIR spectroscopy in measuring important analytical compounds in South African wines was evaluated as early as 1987, when a filter NIR instrument was used to quantify ethanol in wine [32]. Subsequently, the utility of FT-NIR in combination with chemometric techniques for quantitative and qualitative applications on South African wines was evaluated on Chardonnay fermented musts [38]. FT-NIR spectra were collected in the 100-2500 nm region, at a resolution of 2.5 nm, using a 0.5 mm pathlength quartz cell. The percentage sugar and free amino nitrogen (FAN) values in the grape musts were determined, while FT-NIR and SIMCA (soft independent modelling of class analogy) was used to discriminate between Chardonnay samples (*n* = 107) in terms of their malolactic fermentation status and ethyl carbamate content.

Monitoring of grape quality in the vineyard during ripening and at harvest at the weighbridge was performed using FT-IR spectroscopy in the region 929-5011 cm^{-1} on a WineScan instrument [27]. Partial least squares calibration models, using independent test set validation, were developed to quantify total soluble solids (TSS, expressed as ºBrix), pH and titratable acidity (TA, expressed as g/L tartaric acid). With this work, the objective was to establish rapid, high-throughput and low-cost analytical methods for monitoring grape quality in an industrial South African cellar with an annual intake of about 105,000 tons of grapes and producing in excess of 75 million litres of wine [27]. Fourier transform infrared spectra of freshly pressed grapes (*n* = 1170) were collected in transmission mode over three vintages, 2005-2007. The average prediction error, referred to as standard error of prediction (SEP), was expressed in the same units as the reference measurement and calculated as described elsewhere [20]. The regression statistics obtained for TSS (*n* = 647 grape juice samples) were SEP = 0.34 ºBrix, r^2 = 0.99 and residual predictive deviation (RPD) 9. The prediction of pH had an average error of 0.04 units, r^2 = 0.95 and RPD 5. The models developed for TA gave average prediction errors of 0.51 g/L, r^2 = 0.96 and RPD 5. The RPD criterion was proposed to evaluate the calibration model [39]. An RPD value of <3 could be considered as an indication that the calibration model is unsuitable for accurate quantitation, a value of 3-5 indicates that the model is suitable for screening, and a value of >5 indicates that the model is suitable for quantitation.

Fourier transform mid infrared spectroscopy has also been used as a tool to rapidly screen the fermentative properties of wine yeasts and to speed up the evaluation processes in the

initial stages of a yeast strain development programme. This work was aimed at the isolation of yeast strains that produce elevated levels of glycerol [40]. The progress of the fermentations could clearly be seen in FT-MIR spectra obtained during the course of the fermentations. Partial least squares models for the quantitation of volatile acidity, glycerol, ethanol, reducing sugar and glucose concentrations in fermented Chenin blanc and synthetic musts were derived from the FT-IR spectra of small-scale fermentations. The accuracy of quantitation of volatile acidity in both wine and must was excellent, with root mean square error of prediction (RMSEP) values of 0.07 g/L and 0.08 g/L, respectively. Root mean square error of prediction in wine and musts for ethanol were 0.32% v/v and 0.31% v/v, and for glycerol 0.38 g /L and 0.32 g/L. For glucose, the RMSEP values were 0.56 g/L in Chenin blanc and 0.39 g/L in synthetic must. These results showed that FT-IR spectroscopy could be used as a rapid low-cost screening method in biotechnological applications.

Fourier transform infrared ATR spectroscopy was also evaluated for its ability to differentiate 11 *Brettanomyces bruxellensis* strains isolated from red wines [41]. The genetic diversity of the strains was determined by restriction endonuclease analysis pulsed field gel electrophoresis (REA-PFGE). These fingerprints were then compared to the FT-IT ATR fingerprints of the whole bacterial cells as well as the FT-MIR spectra of experimental wines produced through contamination with these strains. Results showed the potential of FT-MIR ATR spectroscopy as a complementary method to molecular typing techniques.

A study towards authentication of South African young cultivar wines was performed using FT-MIR spectroscopy, GC and multivariate data analysis [42]. The volatile composition and FTMIR spectra both contributed to the differentiation between the cultivar wines. The best discrimination model for the white cultivar wines, Chardonnay and Sauvignon blanc was based on FTMIR spectra (98.3% correct classification) while a combination of spectra and volatile compounds (86.8 % correct classification) was best to discriminate between the red wine cultivars, Pinotage, Merlot, Shiraz and Cabernet Sauvignon.

4.2.2. Atomic spectroscopy

Atomic spectroscopic techniques are most often used for the determination of the mineral content of wines. Applications of flame atomic absorption spectroscopy (AAS) [43] and electrothermal AAS [44] for metal analysis in wine have been reported. Aside from regulatory analyses, geographical authenticity of wines may be established by a combination of multi-elemental analysis of wines and their provenance soils, and multivariate statistical methods. For example, Coetzee *et al*. [45,46] described a fingerprinting technique for classification of

South African wines according to geographical origin based upon elemental composition. The method is based on the assumption that provenance soil is a primary contributor to the trace element composition of wines. A total of 40 elements were determined using inductively coupled plasma – mass spectrometry (ICP-MS), 20 of which carried geographic specific information, and these were used in statistical methods. A very high success rate was achieved for classification of these wines from three distinct geographical origins. In another study, the elemental composition of wines and their provenance soils from four wine producing regions of South Africa was also used to classify the wines and soils according to geographical origin. Principal component analysis was used to identify relevant variables, while a linear discriminant analysis (LDA) procedure of the identified variables showed a correlation between the elemental composition of the wines and their provenance soils. This relationship is an important pre-requisite for establishing a fingerprinting methodology [47]. Quadrupole-based ICP-MS was also used to determine the isotope ratios of $^{11}B/^{10}B$ and $^{87}Sr/^{86}Sr$ of wines and soils of four major South African wine-producing regions and to establish a fingerprint for origin verification of the wines. The $^{11}B/^{10}B$ ratios were used to discriminate between origins and, together with the concentrations of selected elements, used as independent variables in linear discriminant analysis, yielded a highly successful method for classification of geographical origins. A good correlation between B and Sr isotope ratios and the provenance soil was found, but the $^{87}Sr/^{86}Sr$ ratios showed limited potential as indicators of origin [48].

4.3. Chromatography

Despite the power of spectroscopic techniques for the high-throughput analysis of a wide variety of compounds in wine samples, many applications in grape and wine analysis require separation of individual chemical species. In many instances, spectroscopic methods do not provide the required selectivity and/or sensitivity for the analysis of specific compounds in the wine matrix. This is especially true for the complex organic fractions of wine, such as the volatile compounds, phenolics and important trace-level constituents.

By far the most common chromatographic methods used for wine analysis are GC and high performance liquid chromatography (HPLC). The application of these and other separation methods vary between routine quantitation of constituents and in-depth investigation of wine chemical composition. In the latter type of research, advances in instrumentation continue to be used to obtain more detailed chemical information, especially using hyphenated techniques such as gas chromatography – mass spectrometry (GC-MS), liquid chromatography – mass spectrometry (LC-MS) and advanced spectroscopic detection

systems such as tandem MS instruments, NMR spectrometers, etc. In fact, the continuous development of new methods has revolutionised our understanding of wine chemistry and ageing, and further developments in this field are essential for quality control purposes as well as for obtaining a more detailed knowledge of the chemistry of grapes and wine.

In the following sections, gas and liquid phase separations will be discussed separately in terms of their applications to wine analysis in the African context. In much of the research reported here, sample preparation and advanced statistical analysis play important roles, in conjunction with the separation methods, and these aspects will also be addressed where relevant.

4.3.1. Gas phase separations

Wine volatiles comprise of a diverse range of chemical molecules with concentrations spanning a few orders of magnitude. To date more than 800 volatiles have been identified in wine. In terms of the analysis of these compounds, the vast majority of research focus has been on the determination of the base wine aroma compounds comprising the so-called major volatiles, which include the principal fermentation derived esters, alcohols and acids. Analysis of these compounds is routinely performed using generic GC methods combined with flame ionisation detection (FID) and more recently MS detection. On the other hand, for the analysis of specific odour impact compounds, various dedicated extraction, separation and detection techniques have been described. Examples of these compounds include terpenes, volatile phenols, sulphur compounds, norisoprenoids, pyrazines, etc.

Modern developments in gas phase separation technologies, such as the progression from wide-bore packed columns to capillary columns, have played a vital role in the expansion of analytical possibilities for wine analysis. Further important developments in sample pre-treatment procedures and more sensitive and selective GC detectors have been influential in extending the application of GC for analysis of wine volatiles.

Major volatiles: Early work on wine volatiles employed packed-column GC separation. For example, van Wyk *et al.* [49] described for the first time the importance of isoamyl acetate in the distinctive fermentation bouquet of young Pinotage wines. Pinotage is a unique South African cultivar cross-bred from Hermitage (Cinsault) and Pinot noir in 1925. These authors reported a clear correlation between quantities of isoamyl acetate and the characteristic aroma attributes of young Pinotage wine, which decreased with ageing as the levels of this constituent declined. Houtman *et al.* [50] quantified two acetate and three ethyl esters in

South African grape juice and wine to identify the most important factors influencing ester production during wine fermentation. No noticeable differences between grape cultivars were observed. In 1981, Marais *et al.* [51] used a packed column GC to quantify 16 major volatile constituents in Pinotage and Cabernet Sauvignon wines. The data were used in combination with discriminant analyses to differentiate between the wines according to cultivar and geographical origin. The importance of isoamyl acetate levels in the differentiation between Pinotage wines was once again highlighted.

With the advent of capillary GC, the number of compounds that can be separated and quantified in a single analysis increased significantly. Of the vast variety of stationary phase coatings available for fused silica capillary columns, the preferred phases for separation of wine volatiles are polyethylene glycol (PEG) or 'WAX' phases. Nitroterephtalic acid modified PEG phases (free fatty acid phases, FFAP) has also been used extensively due to the reduced peak tailing observed for polar analytes on these columns (especially relevant in the case of grape and wine volatiles). On the other hand, non-polar phases such as polydimethylsiloxane (PDMS) are preferred for the analysis of specific classes of apolar compounds such as terpenoids and volatile phenols [52,53], while dedicated phases such as the PDMS-based SPB-1 sulphur phase have been used for the analysis of sulphur compounds [54].

In combination with liquid–liquid extraction (LLE), typical routine capillary GC-FID methods enable the quantitation of 20 to 50 acids, alcohols and esters. Freon was extensively used for the extraction of major volatiles in the past [51,52,55,56], although this has largely been replaced by more environmentally friendly solvents. For example, using diethyl ether LLE Louw *et al.* [57] reported the concentrations of major fermentation derived aroma constituents in 925 young single cultivar South African wines. These data were used to study the variation in volatile concentrations between cultivars and vintages, as well as to derive classification models for the identification of individual cultivars. Several other studies have used data for major volatiles to differentiate between South African wines according to cultivar [58,59] and vintage [60]. Furthermore, major volatile data in combination with FT-MIR have been used to discriminate between South African young cultivar wines according to grape variety using multivariate data analysis methods [42].

Gas chromatographic data have in recent years been employed extensively in biotechnology research related to grapes and wine [61,62]. Intensive research has focussed on the importance of wine yeast on the flavour properties of wines and derived products [63-65].

For example, the effect of esterase activity [66,67] and branched-chain amino acid transaminase activity [68] on wine flavour profiles has been investigated. Yeast strain selection for wine and brandy production is also partially based on the volatile profiles of these products [69,70]. Furthermore, GC data are extensively used in metabolomic [71] and molecular biology [72] yeast research. Generic GC-FID data for major volatiles are typically used to relate volatile content to the biological aspect under investigation [66-69,71].

Other volatile compounds: In addition to the analysis of major volatiles, significant GC research in recent years has focussed on the determination of specific minor volatile constituents. These generally include impact odourants which are present at low levels in the wine matrix, and therefore dedicated methods are required for their determination. Methods of analysis for trace-level compounds therefore also often require selective extraction and pre-concentration techniques and/or selective detection strategies.

For example, Zietsman *et al.* [72] reported a method for the analysis of wine terpenoids in order to study the effect of co-expression of selected glucosidase and furanosidase genes in *Saccharomyces cerevisiae* to release free monoterpenoids. For the analysis of wines, a C18-based solid phase extraction (SPE) procedure was developed which allowed pre-concentration of the extract prior to analysis by GC-FID on an FFAP column.

Acrolein (2-propenal) is a toxic compound formed from 3-hydroxypropionaldehyde. It has been implicated in the formation of bitterness in wines [73]. The determination of this compound is therefore important, although its reactivity complicates the analysis [74]. For the analysis of acrolein in various matrices, derivatisation is often employed, although methods for the analysis in wine using solid phase micro extraction (SPME) and sample enrichment probe (SEP) [75] extraction have been reported [74].

The volatile phenols 4-ethyl phenol, 4-ethyl guaiacol, 4-vinyl phenol and 4-vinyl guaiacol are known to originate from wood ageing, but elevated levels of these compounds are also associated with *Brettanomyces* spoilage. Smit *et al.* [52] employed LLE using Freon 113 for the extraction of three volatile phenols in Weisser Riesling wines prior to their determination by GC-MS in scan mode. This method was used to study the effect of expressing various phenolic acid decarboxylase genes in *Saccharomyces cerevisiae.* The volatile phenols *o*- and *p*-cresol, phenol, ethyl guaicol, 2,6-dimethoxyphenol and guaicol, together with other wood-derived volatiles including fufural derivatives and lactones, were analysed in pot-still brandies by GC-FID on a WAX column [76-78].

Volatile thiols are influential aroma constituents, which may contribute positively or negatively to wine flavour. The analysis of these compounds is challenging due to their low concentrations and reactivity. Several highly volatile sulphur compounds such as methanethiol, dimethyldisulphide, dimethyltrisulphide and hydrogen sulphate are generally associated with off-flavours. The analysis of these compounds by large volume headspace injection using a programmed temperature vaporisation (PTV) injector and GC analysis in combination with selective pulsed flame photometric detection (PFPD), has been reported by Knoll *et al.* [54]. The sulphur compounds 4-mercapto-4-methylpentan-2-one and 3-mercaptohexan-l-ol and 3-mercaptohexyl acetate contribute to varietal aroma of for example Sauvignon blanc wines. In order to study the production of these compounds, Swiegers *et al.* [64] used stable isotope dilution analysis (SIDA) in combination with headspace (HS) SPME-GC-MS.

1,1,6-Trimethyl-l,2-dihydronaphthalene (TDN) is a potent aroma compound in wine. This compound may be partially responsible for the typical bottle-aged kerosene character of aged Riesling wines and has an odour threshold value of 20 µg/L. It has been analysed by GC-MS in selected ion monitoring (SIM) mode following acid hydrolysis of the precursors isolated from wine by HPLC and thin layer chromatography (TLC). The glycosidic precursors of TDN in Riesling wines were structurally elucidated in these preparative fractions by means of NMR [53]. The determination of this compound by comprehensive 2-dimensional gas chromatography in combination with time-of-flight MS detection (GC×GC-TOF-MS) in South African wines has also been reported [79,80].

The varietal aroma compounds in *Vitis vinifera cv.* Khamri grape juice, a native variety from Tunisia, were investigated by Souid *et al.* [81]. These included a number of higher alcohols, terpenes, acids, phenols and norisoprenoids. For the analysis of these diverse compounds, GC-FID and GC-MS were used, while gas chromatography-olfactometry (GC-O) was used to investigate the aroma profile of the juice. The authors fractionated the grape juice volatiles using SPE into free and bound fractions. The bound volatiles were enzymatically released prior to their analysis [81].

Sample preparation for wine volatile analysis: For the analysis of wine volatiles, sample preparation represents an especially important step in the analytical process. Effective extraction and pre-concentration of volatile constituents from the aqueous wine matrix is essential for their accurate qualitative and quantitative analysis. The choice of sample pre-

treatment technique depends on the goals of the analysis. For the analysis of major volatiles, for example, LLE extraction is most often employed due to the relative simplicity and low cost of the technique. Extraction using Freon as solvent was previously utilised extensively [51,52,55,56], although in recent years environmental concerns have largely resulted in the phasing out of its use. The use of diethyl ether in particular for the extraction of major volatiles has also gained widespread application [42,49,50,57,65-69,71], although other solvent mixtures such as pentane/dichloromethane (2/1) have also been utilised [81].

On the other hand, important developments in sample preparation techniques have proved indispensable, especially for the detection of low level odour active constituents, and have also significantly broaden the range of compounds that can be determined in a single analysis for untargeted methods. Sample pre-treatment methods which have gained widespread acceptance as powerful alternatives to conventional LLE for wine volatile analysis include SPE and various solventless sorptive extraction methods such as solid phase micro extraction (SPME) and stir bar sorptive extraction (SBSE).

Sorptive extraction techniques: Sorptive extraction techniques such as internally coated open tubular traps (OTTs), SPME and SBSE have been shown to be advantageous for the extraction of volatiles from complex matrices such as wine. Sorptive extraction is based on the partitioning of chemical constituents into a liquid stationary phase. This approach provides several benefits compared to conventional extraction methods such as LLE, including elimination of the use of (often toxic) solvents, higher sensitivity and easy automation. The most common phase used in sorptive extraction is PDMS due to its well-known advantages of high temperature stability and inertness. Note though that in the case of some phases used in SPME (for example PSDVB or Carboxen phases), analyte retention is due to adsorption rather than sorption.

Open tubular traps involve the use of a tube coated with a thick layer (up to 12 µm) of PDMS. The application of OTTs in both headspace and immersion modes has been demonstrated. The sample is typically sucked or pumped through the trap until breakthrough occurs. The trapped analytes are subsequently eluted using a solvent, or thermally desorbed prior to GC analysis. Burger and Munro demonstrated the applicability of OTTs for wine analysis as early as 1986 [82]. OTT was used for the headspace extraction of volatiles in Gewürztraminer and Crouchen blanc wine, although no specific compounds were identified [82].

Solid phase micro-extraction (SPME) involves the use of a fused silica microfiber coated with the extraction phase (a wide variety of sorbent or adsorbant phases and mixtures is nowadays commercially available). The fibre is fixed to the stainless steel plunger of a syringe, allowing easy exposure or retraction of the fibre. Depending on the nature of the analytes, headspace or immersion SPME is possible. Following extraction, the fibre is typically inserted in a hot split/splitless injector and exposed to introduce the analytes to the chromatographic column. SPME, most often used in the headspace mode (HS-SPME), utilising a variety of stationary phases has been shown to be ideally suited for the extraction of volatiles from wines. For example, Weldegergis *et al.* [80] used a carboxen/polydimethylsiloxane (CAR/PDMS) SPME fibre in the headspace mode for the extraction of volatiles from South African Pinotage wines prior to analysis by GC×GC. Time-of-flight mass spectrometry was used to identify a large number of volatile compounds, including major and minor constituents such as esters, alcohols, acids, aldehydes, ketones, acetals, terpenes, furans and lactones. Furthermore, volatile sulphur compounds as well as nitrogen containing constituents (notably methoxypyrazines) were also detected, clearly illustrating the utility of SPME when used in combination with highly sensitive detectors. More recently a similar methodology using HS-SPME-GC×GC-TOF-MS was used for the analysis of Pinotage wines submitted to malolactic fermentation [83]. In this case a DVB/CAR/PDMS fibre was used, although in general similar compounds were identified in both studies [79,83]. Significant research activity has focussed on developing novel phases for SPME. For example, Wan Ibrahim *et al.* [84] developed a new sol–gel hybrid polydimethylsiloxane-2-hydroxymethyl-18-crown-6-coated fibre for the extraction of low levels of organophosphorous pesticides from a diverse number of fruits, including grapes.

Stir bar sorptive extraction (SBSE), developed by Baltussen *et al.* in 1999 [85], involves the use of a magnetic stir bar that is encapsulated in a glass sleeve and coated with PDMS. The stir bar is introduced into the aqueous sample and sorptive extraction occurs whilst stirring. Extracted analytes are subsequently thermally desorbed for GC analysis. Similar to SPME, sampling can also be performed in the headspace, referred to as head space sorptive extraction (HSSE). Varying amounts of PDMS can be used in SBSE, typically ranging between ~50-200 µL. The higher amount of stationary phase is responsible for the higher sensitivity of SBSE compared to SPME. However, unlike SPME where a wider range of phases may be used, PDMS is currently the only commercially available phase for SBSE.

The application of SBSE in immersion mode for wine analysis was first demonstrated by the extraction of dicarboximide fungicides by Sandra *et al.* [86]. Thereafter, several applications

for the extraction of mostly major volatiles and semi-volatiles from wines were reported. Tredoux *et al.* [58] utilised the technique, also in immersion mode, for the extraction of major volatiles, volatile phenolic compounds, furan derivatives and some minor volatile constituents such as aldehydes, ketones and lactones. These volatile data were used to classify white and red South African wines according to cultivar. Furthermore, the application of HSSE for the quantitative analysis of volatiles in young South African red and white wines has been demonstrated [87]. The compounds quantified comprised a number of major volatiles as well as some wood-derived compounds such as oak-lactones, vanillin and volatile phenols [87]. This validated HSSE method was also used in combination with multivariate statistical methods to classify South African wines according to cultivar [59]. Pinotage wines, in particular, were clearly differentiated by higher concentrations of isoamyl acetate and ethyl octanoate.

Solid phase dynamic extraction (SPDE) is an alternative sorptive extraction technique where the PDMS trapping phase is coated on the wall of the needle of a headspace sampling syringe. Analytes are sampled in the headspace, followed by thermal desorption and large volume injection. This technique was used by Malherbe *et al.* [88] to investigate the volatile profiles of fermenting grape musts in problem fermentations. These authors reported the determination of a significant number of major volatiles, together with some minor constituents including several potentially odour active esters, terpenes and norisoprenoids.

Solid phase extraction: SPE is based on the extraction of volatile compounds from aqueous solutions using a suitable stationary phase. For wine volatiles, C18 and polystyrene-divinylbenzene (PSDVB) phases are most commonly used. The high capacity of these cartridges imply that large pre-concentration factors may be achieved by SPE, while the careful selection of suitable rinsing and eluting solvents may be used to selectively extract certain classes of compounds.

A simple SPE method based on a C18 phase was used by Zietsman *et al.* [72] to extract and pre-concentrate free monoterpenes from wine prior to GC-FID analysis on an FFAP column. The procedure entailed rinsing the cartridge with water following sample loading, and subsequent elution of the volatiles using dichloromethane.

Souid *et al.* [81] reported an interesting SPE procedure based on a PSDVB phase for the fractionation of Tunisian grape juice volatiles. Free aroma compounds were eluted from the cartridge using dichloromethane, whereas the bound volatiles were eluted with ethyl acetate.

This fraction was subsequently submitted to enzymatic hydrolysis followed by LLE with pentane/dichloromethane. These fractions were analysed by GC-FID, GC-MS and GC-O in order to in establish the aroma profile of the native Tunisian grape variety *Vitis vinifera cv.* Khamri [81].

Solid phase extraction (SPE) has also been used as alternative to SPME for the analysis of volatiles in South African wines by GC×GC-TOF-MS. The authors used an SPE method based on that of Campo *et al.* [89] to selectively remove the more polar major volatiles using an aqueous rinsing solvent consisting of 50% (v/v) methanol and 1% $NaHCO_3$. The authors demonstrated that this sample pre-treatment procedure proved much more suited for the analysis of apolar high-boiling compounds such as terpenes, volatile phenols, lactones and sulphur compounds [79].

Derivatisation of wine constituents: Derivatisation is often used to modify non-volatile or highly polar chemical compounds not otherwise amenable to GC analysis [90]. For example, Jolly *et al.* [70] used methylation of fatty acids prior to their analysis by GC-FID (note that underivatised fatty acids may nowadays also be analysed on FFAP columns).

Especially in metabolomics research, derivatisation prior to GC analysis is frequently applied [91]. For untargeted screening of wine or grape metabolites, including polar and high molecular weight compounds such as sugars, long chain fatty acids, amino acids, etc, trimethylsilyl derivatisation is often used. Grimplet *et al.* [92] employed a trimethylsilyl derivatisation protocol using N-methyl-N-trimethylsilyltriflouroacetamide together with trimethylchlorosilane as derivatisation reagents for the determination of grape and fermentation derived metabolites such as amino and organic acids, phenolic compounds and sugars. In related research, Ali *et al.* [93] recently investigated the stereochemistry of wine amino acids with the objective of establishing a method for wine age authentication. The time-dependent conversion of *L*-amino acids into the *D*-form follows first-order kinetics, with the result that the extent of enantiomerisation may reveal the age of a wine. Amino acid enantiomers were determined by chiral GC-MS in selected ion monitoring (SIM) mode following ion-exchange based sample clean-up and derivatisation to yield the N-(O)-pentafluoropropionyl-2-propyl esters. Although the presence of *D*-enantiomers was established in aged wines, no correlation was evident between these stereochemical forms and product age.

Comprehensive 2-dimensional GC: While conventional capillary GC has proven to be an indispensable tool in the routine analysis of volatiles associated with wine aroma, these methods do show some limitations in terms of resolving power and dynamic range when complex mixtures such as wine are analysed. Comprehensive two-dimensional GC (GC×GC) provides a powerful alternative method capable of providing much higher separating power. This is achieved by exploiting the use of two stationary phases to combine separation based on boiling point and polarity. In recent years, GC×GC has also been applied to wine analysis in Africa. Weldegergis *et al.* [80] used HS-SPME-GC×GC-TOF-MS for the detailed investigation of South African Pinotage volatiles. This approach allowed the identification of a much larger number of compounds compared to 1-dimensional GC: 48 compounds were identified using standards, while a further 158 compounds were tentatively identified using a combination of linear retention index (RI) data and deconvoluted mass spectra obtained by TOF-MS. Compound classes identified included esters, alcohols, aldehydes, ketones, acids, acetals, furans and lactones, sulphur compounds, nitrogen compounds, terpenes, hydrocarbons and volatile phenols. Subsequently, the same group extended this research by using SPE pre-treatment in combination with GC×GC-TOF-MS analysis [79]. By removing the more polar major volatiles, the identification of trace-level, high-boiling apolar odourants such as terpenes, lactones and volatile phenols was facilitated. Figure 4.4 presents an example of a contour plot obtained for the analysis of a South African Cabernet Sauvignon wine, 214 compounds were tentatively identified in this study, while an additional 62 compounds were positively identified using standards. Another recent report on GC×GC-TOF-MS demonstrated the applicability of this technique for semi-quantitative analysis of wine volatiles [83]. In this study, HS-SPME-GC×GC-TOF-MS was used to investigate the volatile composition of Pinotage wines submitted to malolactic fermentation using different lactic acid bacteria strains. Excellent differentiation was obtained using data obtained by GC×GC, which allowed identification of the volatile compounds responsible for the variation between the wines produced with the different starter cultures.

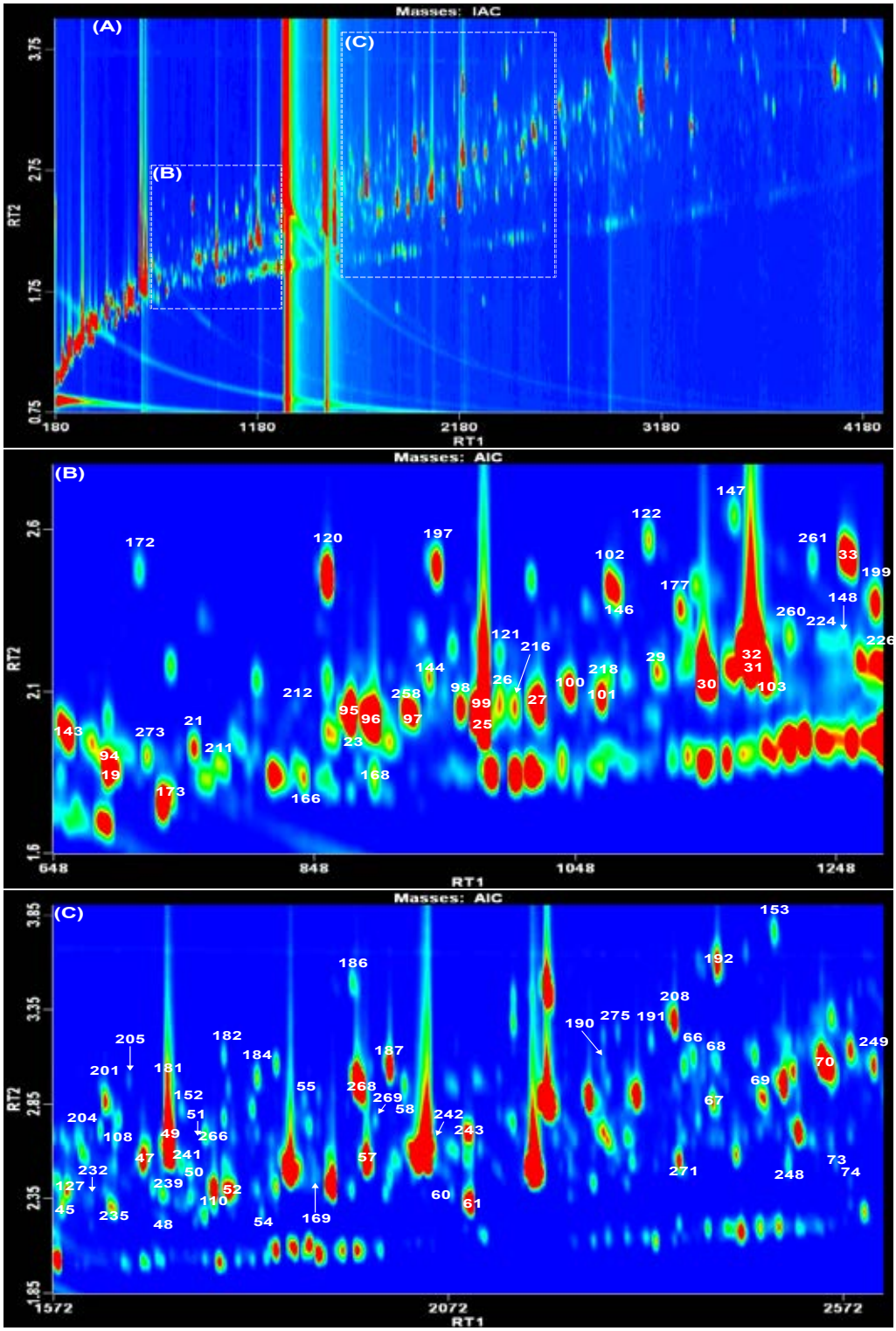


Figure 4.4. (A) Example of a contour plot obtained for the SPE-GC´GC-TOF-MS analysis of a South African Cabernet Sauvignon wine. (B) and (C) present detailed portions of the contour plot to illustrate separation of volatile compounds. The column set used in these experiments comprised of a 30 m ´ 0.25 mm i.d. ´ 0.25 µm df Rxi – 5Sil MS primary column coupled to a 0.8 m ´ 0.18 mm i.d. ´ 0.18 µm df Rtx – PCB secondary column. Reprinted with permission from [76].

4.3.2. Liquid based separations

Many wine and grape constituents are not amenable to gas-phase separations due to either limited volatility and/or thermal stability. For the separation of these compounds, liquid chromatography is the separation method of choice, although capillary electrophoresis (CE) has been applied as an alternative liquid-based separation technique. Aside from routine regulatory analyses (see Section 4.4), which are often performed using classical wet chemistry methods, the application of HPLC for the analysis of a variety of wine constituents has been growing tremendously. Advances in columns and instrumentation have contributed to a significant increase in the number of non-volatile wine constituents which may be accurately quantified or identified using HPLC.

4.3.2.1. High performance liquid chromatography

Liquid chromatographic methods for wine and grape analysis can roughly be divided into one of two types. In the first instance, routine methods are used for the quantitative analysis of wine constituents for regulatory purposes, as well as to monitor the production process. In this case, the emphasis is on simplicity, speed, robustness and quantitative accuracy, and as a rule simpler instrumental configurations such as HPLC with UV or fluorescence detectors are used for this purpose. These instruments are relatively cheap and robust, and therefore ideally suited for routine analyses in wine laboratories. On the other hand, for the detailed chemical investigation of complex, low-level constituents with the aim of investigating wine and grape chemistry and elucidating new constituents, advanced LC-MS methods are most often used. For each of these methods, but especially for the second type, sample preparation plays an essential role. In the following discussion, an overview of the application of HPLC for wine and grape analysis in Africa is presented, with the discussion structured according to chemical classes.

Organic acids and sugars: Organic acids affect the taste and mouth-feel of a wine, enhance colour stability, limit oxidation and together with ethanol, are largely responsible for the microbial and physicochemical stability of table wines [94,95]. As primary substrates during alcoholic fermentation, sugars are responsible for the formation of ethanol as well as a number of secondary products, and their concentrations are used to determine the endpoint of fermentation. The levels of glucose and fructose, the major hexoses present in grapes and must, are used to determine optimal grape ripeness. While high throughput methods (see Section 4.1) are typically used for the determination of acids and sugars in grapes and wine for regulatory purposes, more selective methods are required for an in-depth knowledge of the organic acid and sugar composition.

For this purpose, HPLC is the preferred chromatographic method. Use of standard reversed-phase (RP) columns for organic acid analysis dictates aqueous mobile phases, whereas RP analysis following derivatisation offers the advantages of better chromatographic performance and improved (more sensitive and selective) detection, although at the cost of increased method complexity. However, ion exclusion HPLC, where separation is achieved through a combination of ion exclusion and partitioning processes using dilute acidic mobile phases, is the method of choice for organic acid analysis using low-wavelength UV and refractive index (RI) detection [54,65,71,96,97]. These methods have the added advantage of simultaneously allowing the measurement of the major wine sugars (fructose and glucose) as well as glycerol and ethanol.

For sugar analysis, aminopropyl or equivalent polar columns may be used in combination with water/acetonitrile mobile phases [97] in hydrophilic interaction chromatography (HILIC) mode. Ultraviolet detection has been employed under these conditions, although the use of RI or evaporative light scattering detection (ELSD) is more common. Compared to RI, ELSD offers increased sensitivity and gradient compatibility, although this type of detector commonly produces non-linear calibration curves and gradient dependent response [98].

When using ion-exclusion chromatography, direct injection of especially red wines results in co-elution and poor integration precision when using non-selective UV detection at 210 nm. One of the drawbacks of ion-exchange methods is the limited scope for tuning the selectivity of organic acid analysis. Changing the pH does not greatly affect the retention of the earlier eluting compounds, and addition of organic solvent leads to an undesirable decrease in retention. To overcome these limitations, de Villiers *et al.* [97] reported a sample clean-up procedure using SPE on PSDVB cartridges for the simultaneous analysis of organic acids and sugars in wine. A low pH was used to ensure retention of phenolic compounds (including phenolic acids) on the cartridge, while organic acids and sugars were eluted with 20 mM sulphuric acid. This procedure allowed interference-free analysis of organic acids (by ion-exclusion on an Aminex phase) and sugars (by HILIC-ELSD) in the same sample [97].

Biogenic amines: Biogenic amines are primarily formed by decarboxylation of amino acids, and are known to have physiologically detrimental effects if present in sufficient amounts. Lactic acid bacteria are largely responsible for the production of bio-amines in wine. For a recent review on biogenic amines in wine covering the factors affecting formation, analytical and molecular detection methods and how to control their production in wine, the reader is

referred to [99]. Analysis of amines in wine has been performed by GC and CE, although HPLC is more commonly used, often in combination with the analysis of amino acids. A variety of derivatisation reactions has been employed in combination with UV or fluorescence detection [99]. Cilliers and van Wyk [100] used an HPLC method with derivitisation to quantify the histamine and tyramine content of South African wines. Sample clean-up involved cation-exchange on an Amberlite CG-120 cartridge, followed by derivitisation with o-phthaldehyde prior to RP-LC analysis with fluorescence detection. The content of histamine and tyramine in 184 and 156 South African wines, respectively, was found to be similar to that reported for wines from other countries [100].

Chlorophylls and carotenoids: These photosynthetic pigments are of importance not only in the grapevine leaves, but also in grape berries as precursors for the production of norisoprenoids, which are known to be significant contributors to wine aroma. Lashbrooke *et al*. [101] reported an extensive study on the optimisation of a single-step extraction procedure for carotenoids and chlorophylls in grapevine leaf and berry tissue. Special attention was given to optimisation of extraction parameters in order to avoid degradation of the nine target analytes. Extracts were subsequently analysed by RP-LC with UV/Vis detection, which allowed the accurate monitoring of the carotenoids and chlorophylls in grape berries and leaves as a function of the different stages of ripening [101].

Phenolic compounds: Phenolic compounds are very influential constituents of grapes and wine. Phenolics affect organoleptic properties through their contribution to astringency, bitterness and colour. Furthermore, phenolics play an important role in the ageing of wines, as well as in grape browning. Finally, several important health benefits associated with modest consumption of especially red wine have been ascribed to the phenolic content of these products, which is partially responsible for the significant research activity in the field of wine phenolics.

In line with increased scientific interest in phenolic composition in wines in recent years [102], much African research into this influential class of wine constituents has also been performed. Liquid chromatography is most often employed to study wine and grape phenolics. RP-LC on C18 or equivalent stationary phases is virtually exclusively used for phenolic analysis. For quantitation of the principal wine phenolics, UV detection is commonly employed, while the recent trend has been increasing application of MS for structural elucidation and quantitation (the latter commonly using MS/MS) purposes. Due to the complexity of wine phenolics, extensive pre-fractionation is also often employed. In addition,

in the past 5 years influential developments in HPLC, primarily in terms of the use of smaller particle-packed columns, elevated temperature and multidimensional separations, have also been exploited for these compounds. The application of these developments in HPLC to phenolic analysis has recently been reviewed [103].

Due to the variety and complexity of wine phenolics, a wide range of methods has been applied for their analysis. An extensive comparison of several of these methods for wine phenolic determination was reported by De Beer *et al.* [104]. Techniques compared included liquid chromatographic methods (normal and reversed phase HPLC), several selective chemical reactions for bulk determination of specific phenolic sub-classes (Folin-Ciocalteu, dimethylaminocinnamaldehyde, tannin, polymeric pigment and antioxidant assays) as well as cyclic voltammetry. Results obtained by each of these methods were compared for different classes of wine phenolics including monomeric phenols and anthocyanins, total phenol content, high molecular weight polymer and total polymer content. Significant correlation was observed for several of these methods for selected sub-classes [104]. The authors concluded that, due the complexity of wine phenolics, a combination of methods should be used for wine polyphenol analyses, with the specific methods selected dependent on the goals of the study (i.e. individual chemical constituents or total amounts of specific sub-classes).

Numerous literature reports provide quantitative data for phenolics in African wines. Goldberg and co-workers reported the concentrations of selected phenolic compounds in a large number of commercial red and white wines from across the globe [105,106]. Compounds were quantified by RP-LC with diode array detection (DAD) using UV spectral matching to confirm identity. For South African red wine cultivars, the levels of quercetin were found to be among the highest for the cultivars studied, while relatively high levels of p-coumaric acid were also found compared to wines from other countries [105]. In a related study, these authors used the same RP-LC-DAD method to determine the concentrations of selected phenolics (quercetin, *p*-coumaric acid, catechin and epicatechin, trans-resveratrol and polydatin) in 644 commercial white wines from all major wine-producing countries, including South Africa, and reported the highest levels of flavan-3-ols in Sauvignon blanc wines from that country [106]. Basha *et al.* reported the comparison of phenolic profiles of muscadine (*Vitis rotundifolia*) and *Vitis vinifera* grape wines from across the globe based on reversed phase HPLC analysis with UV detection at 280 nm [107]. Rossouw and Marais [108] reported the concentrations of 39 phenolic compounds (including non-coloured phenolic compounds and anthocyanins) in 260 South African Pinotage, Shiraz, and

Carbernet Sauvignon wines from four vintages. The RP-LC method employed a PLRP-S polymeric column with diode array detection based on the work of Waterhouse *et al.* [109]. The authors were able to obtain a very good differentiation between the cultivars (independent of vintage) using discriminant analysis based on mean levels of the quantified phenolics [108]. De Villiers *et al.* [110] reported data for non-coloured phenolic content in five red (n = 55) and three white (n = 38) South African cultivars. White wines were directly injected, while an SPE method was used to remove interference from polymeric phenolics for red wines [97]. Quantitative data for 22 phenolics were used to classify the studied wines according to cultivar using multivariate statistical methods (Figure 4.5). In fact, quantitative phenolic data obtained by HPLC have successfully been employed to differentiate between South African wines according to both grape variety [108,110,111] and vintage [108] by multivariate statistical methods in various reports.

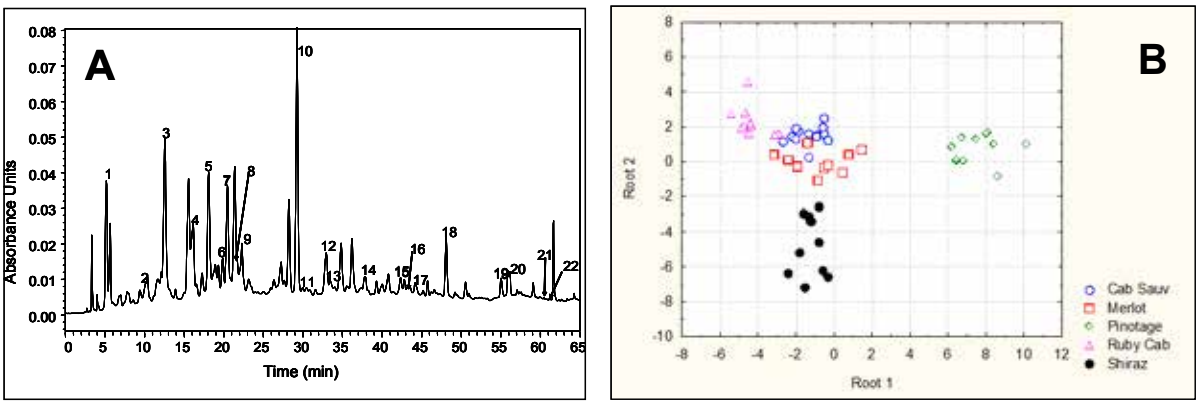


Figure 4.5. (A) Typical HPLC-UV chromatogram obtained for the analysis of South African red wine, illustrating 22 phenolic compounds quantified. (B) Scatter plot of the scores on the first two canonical roots obtained from the quantitative polyphenol data for red wines. Reprinted with permission from [110].

Du Toit *et al.* utilised an LC-UV method on a monolithic column (Chromolith Performance RP-18) to quantify 21 non-coloured phenolics and anthocyanins, as well as polymeric pigments in South African red wines [13]. These data were used to ascertain the effect of micro-oxygenation on the levels of phenolics in the studied wines. In combination with sensory data, it was shown that micro-oxygenation may potentially be used to improve the quality of especially young red wines.

A monolithic column was also used by Liazid *et al*. [112] for the quantitative analysis of 13 non-coloured phenolic in grapes and derived products. A fast (14 minute) method was developed utilising a 100 mm Chromolith Performance RP-18 column operated at 2.5

mL/min in combination with UV and fluorescence detection. The method proved to be reproducible, and its application for the analysis of wine musts was demonstrated.

The stilbene content of wine, especially for the compound *trans*-resveratrol, has received extensive attention in literature due to the beneficial biological activity ascribed to this class of compounds [102]. Guebailia *et al.* [113] reported for the first time the presence of a resveratrol tetramer, called hopeaphenol, in Merlot wines from Algeria. In a more recent paper, the same group also reported for the first time the isolation and characterisation of the resveratrol dihydrodimer *cis*-ε-viniferin from Algerian wine [114]. These compounds were isolated from wine using a combination of column chromatography on a cation-exchange resin, centrifugal partition chromatography using a water/ethanol/ethyl acetate/hexane mobile phase and semi-preparative RP-LC on a C18 column. Following isolation, the compounds were characterised by MALDI-TOF-MS and 1H NMR and 2D correlations [113,114]. Subsequently *trans*-resveratrol, *trans*-piceid, *trans*-ε-viniferin, pallidol, astilbin, hopeaphenol and *cis*-ε-viniferin were quantified in Algerian, Moroccan and Tunisian red and white wines using analytical RP-LC with UV detection. Wines from North Africa were found to contain high levels of resveratrol derivatives [113,114].

The antioxidant properties of wines, and especially the importance of phenolic compounds in this regard [115,116], have been investigated extensively. The role of phenolic compounds in wine as antioxidants has been reviewed by de Beer *et al.* [117]. The same group also reported extensive data on the antioxidant capacity of South African wines. The free radical scavenging activity of South African red and white wines was determined using 2,2'-azinobis(3-ethylbenzothialozinesulfonic acid) radical cations (ABTS) and 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH) [118]. In further work, de Beer *et al.* [119] reported the *in vitro* inhibition of microsomal lipid peroxidation for the major South African red and white wine varieties. Roginsky *et al.* [120] used similar methods in a study which concluded that the antioxidant activity of Californian red wines do not correlate with wine age. Several possible reasons for this rather unexpected observation were discussed [120]. Furthermore, the relationship between the content of individual phenolic compounds and the total antioxidant capacity of Pinotage wines was investigated [121]. Twenty-four individual phenolic compounds (comprising, flavanols, flavonols, anthocyanins and phenolic acids), as well as polymeric phenolics, were quantified using a RP-LC method on a polymeric column [122]. It was found that individual monomeric phenolics were responsible for only a small fraction (11-24%) of the total antioxidant activity of Pinotage wine. The remainder may be ascribed to unidentified compounds (including polymeric phenolics), as well as to synergetic

effects involving phenolic compounds [121]. This same RP-LC method was also used in a study of the effect of oak maturation using different products on the phenolic composition, antioxidant activity and colour of Pinotage wines [123]. While the phenolic content and colour of wines submitted to oak maturation using both traditional and alternative oak products were altered significantly, the total antioxidant activity was found to remain constant [123]. In another study by the same group, the effect of oxygenation on Pinotage phenolic content, colour and antioxidant activity was investigated [124]. The authors concluded that controlled minimal doses of oxygen should be used to avoid detrimental effects in terms of sensory properties and antioxidant activity.

Concerning grape phenolics, Youssef and El-Adawi [125] reported a study on the optimisation of the extraction of Egyptian red grape seed phenolics. RP-LC was used to quantify gallic acid, catechin and epicatechin in the extracts. In other work on proanthocyanidins, Hmamouchi *et al.* [126,127] investigated the phenolic content of four Moroccan varieties of *V. vinifera* leaves. Ten flavonoids were detected in these leaves [126], whilst the oligomeric proanthocyanidin content was found to consist of varying ratios of prodelphinidins to procyanidins [127].

Van Jaarsveld *et al.*, in a series of papers, reported the effect of different wood types, treatments and extraction media to induce rapid ageing of brandy [76-78]. As part of this study, 12 volatile constituents including influential volatile phenols were determined by GC-FID, while a further 10 phenolic compounds were quantified in the extracts by RP-LC with UV detection. These data were correlated with sensory data to determine the highest quality products and relate these to their chemical composition. In general, the best quality extracts contained higher levels of volatile and semi-volatile wood-derived compounds. Better quality products were also obtained using higher concentrations of ethanol as extraction medium [76] and toasted oak [78]. French oak was found to yield initially better quality products, although after 8 months ageing similar results were obtained for the American oak products, while the chemical composition of the products produced from each of these types of oak was found to vary [77].

Anthocyanins: Anthocyanins (anthocyanidin-glycosides) are phenolic compounds responsible for the colour of red grapes and wine. These compounds are also important for their contribution to the health benefits associated with wine consumption and the vital role they play in the ageing of red wines.

De Villiers *et al.* [111] reported a method for the analysis of anthocyanins in five South African red wine cultivars using an RP-LC-UV-MS method. Forty-four anthocyanins and derived products were identified using MS detection and retention times. Sixteen compounds were quantified as malvidin-3-O-glucoside equivalents using selective UV detection, and these data were used to differentiate between the different cultivars. This classification proved to be less effective than that obtained using non-coloured phenolic data [110], at least partially due the fact that a wide range of vintages was studies, and therefore the reduction in levels of free anthocyanins as a function of time affected the differentiation between cultivars.

Ghassempour *et al.* [128] reported a study on the extraction of anthocyanins from Iranian red grape skins by microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE). These authors used RP-LC on C18 columns in combination with UV and MS/MS detection for the quantitation and identification of 9 anthocyanins in the extracts. Both MAE and UAE were found to be suitable methods for the efficient extraction of grape anthocyanins. Choi and co-workers [129] reported the tentative identification of 19 anthocyanins in the skins of *Vitis coignetiae* Pulliat (meoru), a wild vine species native to Korea, by making use of RP-LC-MS/MS. These authors also reported the antioxidant activity of anthocyanins present in the skins.

De Villiers *et al.* [130] reported an investigation into the reversed phase separation of anthocyanins and the factors affecting the efficiency of these separations. The authors demonstrated that the relatively slow inter-conversion (on the same time-scale as the separation) between carbinol and flavylium species in the mobile phase results in relatively broad peaks for anthocyanins under conventional RP-LC conditions [130]. It was further shown how an increase in analysis temperature and decrease of the stationary phase particle size may be used to significantly improve the analysis of red wine anthocyanins [130-132]. The benefits of this approach were exploited by using a 200 mm 1.7 mm phase operated at 50°C in combination with positive mode ESI-MS for red wine analysis [133]. The authors report much improved separation under these conditions, and were able to identify 101 anthocyanins and 36 proanthocyanidins in a single analysis [133].

Reversed phase liquid chromatographic methods based on polymeric C18 phases have been used extensively in the analysis of anthocyanins [121-124]. Oberholster *et al*. [134] used this approach for the quantitation of non-coloured phenolics and anthocyanins in a study related to the mouth-feel of white wines produced with pomace contact and added

anthocyanins. Ristic *et al*. [135] utilised RP-LC on Synergy Hydro-RP and polymeric (polystyrene-divinylbenzene) columns for the analysis of anthocyanins and tannins and flavonols in Australian Shiraz grape skins and seeds and wine to study the effect of shading on grape and wine composition. Several monomeric proanthocyanidins, anthocyanins and flavonols were quantified using these methods, while tannin sub-unit composition was studied using phloroglucinol acid-cleavage. Bindon *et al.* [136] used RP-LC on a Lichrospher 100 RP-18 column for anthocyanin determination in grape berries, while tannins were analysed on a polymeric RP phase according to Peng *et al.* [122]. Changes in anthocyanin composition of berries as a result of partial root zone drying were reported in this study [136].

Non-coloured phenolic compounds such as flavonols may also affect wine colour as a result of chemical reactions involving these compounds during wine ageing [137]. In order to elucidate the pigments formed in this manner, model solutions are often used in combination with advanced analytical methods such as preparative LC, LC-MS and NMR to study reaction products. Es-Safi *et al*. [138-143] have in this manner extensively studied the reactions involving (+)-catechin in model solution. Thus, using HPLC-DAD, LC-MS and NMR spectroscopy, this compound was shown during artificial ageing experiments to first produce colourless dimeric reaction products, followed by the formation of various xanthylium pigments [138,144]. These compounds were also successfully detected in red wine samples by RP-LC-ESI-MS. Furthermore, the reaction between (+)-catechin and glyoxylic acid (the latter produced from oxidation of tartaric acid in wine media), has been studied in model solution. Reversed phase LC with DAD and ESI-MS detection was used to detect the derived products [139,141,145], while 2-dimensional NMR methods such as COSY, TOCSY, ROESY, HSQC and HMBC techniques were used in the unambiguous structural elucidation of each of the reaction products following semi-preparative LC isolation [140]. An overview of the interactions between (+)-catechin and glyoxylic acid and their importance in terms of food organoleptic properties was reported by Es-Safi *et al.* [142].

The same group has extensively studied the reactions between flavonols and anthocyanins with the ultimate goal of elucidating some of the important reactions occurring during wine production and ageing (and in fact fruit-derived beverages in general). Thus the reaction between (epi)catechin and various aldehydes (acetaldehyde, glyoxylic acid, furfural and 5-(hydroxymethyl)furfural) in the presence of mavidin-3-O-glucoside was studied in model solution using a combination of LC-DAD- and LC-MS [146-149]. Both coloured and non-coloured products increasing in size up to tetramers were identified in the reaction mixtures. Further extension of this work involving (+)-catechin and cyanidin-3-O-glucoside in the

presence of furfural and 5-(hydroxymethyl)furfural produced similar pigments, although the formation of coloured products was favoured compared to malvidin-3-O-glucoside. Various bridged oligomeric and polymeric products were identified. The eventual precipitation of some polymeric products may play a role in the reduction in astringency of red wines during ageing [150]. Taken together, this work points to the great diversity of products that may be formed during wine storage and ageing, and the effect that the derived products may have on the organoleptic properties of the product.

In addition, numerous other pathways exist for the transformation of grape-derived anthocyanins in the wine medium [133,137,151]. Fulcrand *et al.* [152] were the first to identify the pyranoanthocyanin products formed by reaction of anthocyanins with pyruvic acid using a combination of HPLC, MS and NMR. Es-Safi *et al.* [153] also investigated the reactions involving malvidin-3-O-glucoside in ethanolic solutions. Using a combination of RP-LC-DAD, HPLC-ESI-MS and one- and two-dimensional NMR analysis of fractions collected by HPLC, these authors successfully characterised two new colourless products formed during storage of malvidin-3-O-glucoside in ethanol [153].

The phenolic content of Pinotage wines has received considerable attention. Characterisation of Pinotage non-coloured and anthocyanin phenolics has been utilised for purposes of classification according to cultivar [108,110,111] and vintage [108], while the antioxidant properties of this wine have been studied in depth [121,123,124]. In addition, this wine has received attention due to high levels of a 4-vinylcatechol adduct of malvidin-3-O-glucoside, referred to Pinotin A [154]. Pinotin A was isolated from Pinotage wines by Schwarz *et al.* by making used of a combination of SPE and high speed counter-current chromatography (HSCCC) [154]. The formation of this compound in wine has been ascribed to the chemical interaction of caffeic acid and malvidin-3-O-glucoside, the principal anthocyanin present in young wines [155]. While not unique to Pinotage wines, the formation of Pinotin A is favoured in wines of this cultivar due to the high content of caffeic acid which is characteristic of Pinotage [156]. Quantitative analysis of this compound obtained by RP-LC analysis for 50 Pinotage wines of vintages 1996-2002 indicated that its concentrations increase up to ~4 years, where after polymerisation or degradation reactions lead to a reduction in concentration [156].

An interesting study involving anthocyanins was reported by Gargouri *et al.* [157], who investigated the binding equilibrium and kinetics of the *Vitis vinifera* enzyme anthocyanidin reductase. A chromatographic method utilising a size exclusion column was used to study

the binding of the enzyme with NADPH, $NADP^+$ and catechin. In this method, the mobile phase contains a fixed amount of potential ligand. By injecting increasing amounts of the ligand with the enzyme, the amount of ligand bound to the enzyme may be deduced from the threshold value where the ligand peak provides zero net absorbance. From these experiments, the dissociation constants for anthocyanidin reductase and the studied ligands could be determined [157].

Miscellaneous: The inherent selectivity of LC-MS/MS has also been exploited for the trace-level determination of methoxypyrazines in South African wines [158]. Although these aroma constituents are normally analysed using GC, LC-MS/MS under optimal conditions was found to provide much lower limits of detection, allowing accurate quantitation of methoxypyrazines in white and red varieties (including the first report on the presence of 3-isobutyl-2-methoxypyrazine in Pinotage wine samples). Multivariate data analysis showed no significant correlation between the levels of methoxypyrazines in 575 South African Sauvignon blanc wines as a function of vintage or geographical origin [158].

Du Toit *et al*. [159] reported a novel LC-MS/MS method suitable for the simultaneous determination of reduced and oxidised glutathione, an important compound in the oxidation of white wines, in grape juice and wine. The method employed an Atlantis C18 phase and positive electrospray ionisation, with detection performed in multiple reaction monitoring (MRM) mode, providing limits of detection in the region of 0.2-0.4 mg/L. This method was used to obtain information on the reduction of glutathione concentrations in South African white wines produced with different levels of oxygen (i.e. reductive, control and oxidative treatments) [159]. More recently, du Toit and co-workers reported the development and validation of an ultra-performance liquid chromatographic (UPLC) method with multi-wavelength UV detection for the analysis of glutathione, catechin and caffeic acid in grape juice and wine [160]. Glutathione was derivatised with *para*-benzoquinone to allow its detection at 303 nm. The use of 2.1 mm internal diameter 1.7 µm RP columns operated at elevated pressures allowed for the development of a rapid analytical method ideally suited for routine analysis and providing significant reduction in solvent consumption [160].

Liquid chromatography – mass spectrometry has also been used in wine-related proteomic research. For example, Rossouw *et al.* [161] utilised an isobaric tag for relative and absolute quantitation (iTRAQ)-based proteomic analysis of two different wine yeast strains at various times during fermentation of a synthetic wine must for the comparative transcriptomic and proteomic profiling of these strains.

Another interesting study utilising LC-MS/MS [162] deals with the (indirect) identification of malvidin in potsherds from archeological sites in Armenia and Syria. Presence of malvidin was established following extraction from the ceramic vessels and SPE sample clean-up followed by alkaline hydrolysis of malvidin to produce syringic acid, which was then detected by RP-LC-MS/MS in MRM mode. The presence of malvidin in some of the potsherds provides supporting evidence for the hypothesis that wine was produced in the Near Eastern highlands around 4000 BC [162].

4.3.2.2. Capillary electrophoresis

Capillary electrophoresis (CE) has, since the early 1990's, received significant attention in the literature as an alternative liquid-based separation method to HPLC. The principal benefits of CE are the inherently high efficiency and speed as well as the versatility of the technique, as reflected in the alternative separation mechanisms offered by the various modes of CE. However, CE methods generally suffer from lower sensitivity and robustness compared to standard HPLC methods, and partially for these reasons the technique has primarily found application in certain niche-areas where it provides clear benefits compared to HPLC (for example chiral separations). Reflecting these trends, CE has also found application in the analysis of grapes and wine, especially for the analysis of compounds not easily determined by HPLC.

The ionic nature of organic acids makes these compounds ideally amenable to CE analysis. The relative mobility difference between acids and other wine constituents is responsible for their separation. As a result, one of the principal advantages of CE for organic acid analysis is elimination of the requirement of sample preparation.

De Villiers *et al.* reported a CE method for the analysis of organic acids in South African wine. The inherent advantages of CE were exploited to allow the separation of the major organic acids in diluted wine. 2,6-Pyridinedicarboxylic acid was used as background electrolyte (BGE) with indirect UV detection. The method was improved compared to previously reported procedures by the addition of ethelenediamine-tetracarboxylic acid (EDTA) to the BGE in order to suppress complexation of citric acid with trace metals present in the capillary. In addition, electrokinetic injection was utilised to avoid problems relating to split peaks associated with pressure injection at high acid levels [163], which was ascribed to differences in the sample and buffer pH [96]. In addition to eliminating the need for sample preparation, the increased efficiency of the method provided more reliable data than HPLC

utilising ion exchange [97] due to less co-elution of other wine constituents (especially problematic in the case of succinic acid in the HPLC method). An example of the analysis of a South African red wine using the developed method is presented in Figure 4.6.

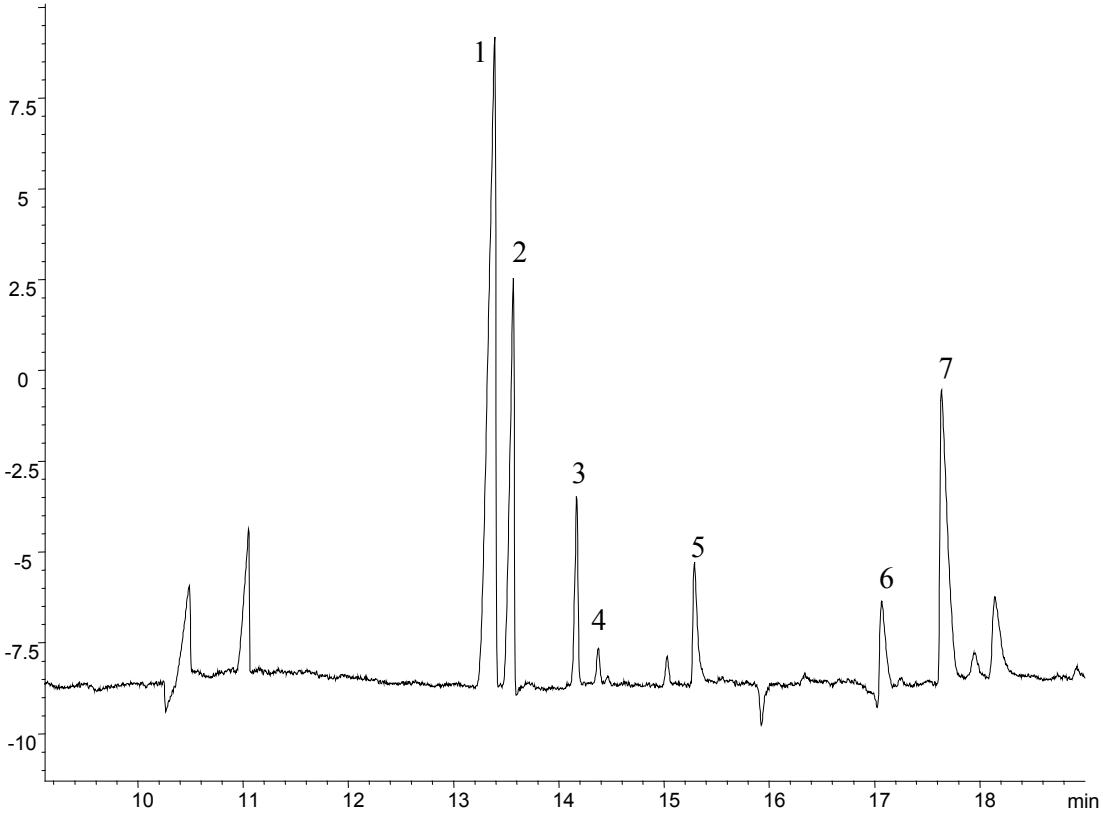


Figure 4.6. CE analysis of organic acids in a diluted South African red wine. Background electrolyte: 7.5 mM PDC, 0.5 mM CTAB, 0.5 mM EDTA, pH 5.6. Capillary: 75 µm i.d., 111.3 cm L_{tot}. Peaks: 1 - formic acid (I.S.), 2 - tartaric acid, 3 - malic acid, 4 - citric acid, 5 -succinic acid. 6 - acetic acid, 7 - lactic acid. Reprinted with permission from [93].

Capillary zone electrophoresis (CZE) has also been used for the analysis of phenolic compounds in South African wines. Vanhoenacker *et al.* [164] compared CZE-UV-ESI-MS with RP-LC-UV-ESI-MS for the analysis of monomeric phenolic compounds in diethyl ether extracts of red wines. These authors concluded the RP-LC remains the method of choice for phenolic analysis. Capillary electrophoresis coupled to MS was found to suffer from poor sensitivity, rendering this technique insufficient for wine analysis. Furthermore, CE has also been applied to detect artificial colourants in red wine (see Section 4.4 for details) [165].

4.4. Regulatory analysis, food safety and quality assurance

In international commerce, laws are passed to regulate the quality, authenticity and health and safety of commodities. The global wine industry is possibly subject to more regulations than most because of the great diversity and complexity of its products. Regulations may cover aspects ranging from how grapes are grown to when and where wine is sold and consumed. In addition to appellation control regulations, national laws regulating this industry are enforced in most countries. Legislation in different countries often differs in terms of the additives and processes allowed in the winemaking process [166]. This has implications for importing/exporting wines between countries, as governed by the relevant trade agreements, and therefore also in terms of the analytical methods required to monitor this industry.

Regulatory laws are primarily concerned with quality, health and safety aspects and generally involve the chemical composition of wines. It should be noted that wine excellence cannot be guaranteed by either objective chemical analysis or the existence of a controlled appellations system. Although sensory evaluations are subjective, and therefore not strictly quantitative by nature, they nevertheless have greater significance than objective chemical analysis alone and play an important role in quality assurance systems (such as the one implemented in South Africa, for example) [94]. Controlled appellation systems largely (but not exclusively) make use of record keeping and inspections for ensuring compliance, whereas chemical analysis is the basis for ensuring conformity to national laws that regulate the wine industry in many countries. In the following discussion, analytical techniques employed in the African wine industry for regulatory, safety and quality assurance will be reviewed.

4.4.1. Regulatory analyses

Despite the extreme complexity of wine, only a few chemical compounds are typically regulated in wine legislation. Regulated wine parameters include alcohol content, reducing sugars, volatile acidity and sulphur dioxide. Analytical procedures for determining these regulated parameters are mostly official methods prescribed by the *Office International de la Vigne et du Vin* (OIV) and are frequently classical wet chemistry methods characterised by high robustness and precision and low cost (the latter is an important consideration in many wine laboratories). Alternative procedures utilising modern, automated instrumental techniques, which provide high sample throughput, sensitivity, selectivity and precision, may also be applied for regulatory analyses. However, for these methods to be endorsed by the OIV, a systematic comparison with the official reference method is mandatory to ensure

suitability [167,168]. In the following sections, a brief overview of the official methods used for regulatory purposes will be presented. Where relevant, more modern methods reported by African scientists will be discussed in more detail.

Alcohol content: The alcohol content of wine is an important parameter that is universally displayed on wine labels and which factors in the calculation of excise duty in commerce. The alcohol content of different types of wine is legislated in many countries. The determination of the wine alcohol therefore needs to be accurate and precise as tolerances in the order of 0.5-1.0% of the documented value are typically enforced. Procedures for the determination of wine alcohol content may be divided into methods that measure the physical characteristics of a solution (typically the distillate of a wine) and those based on the chemical properties of ethanol. Chemical methods include dichromate oxidation and enzymatic determination, while physical methods use specific gravity or boiling point depression. The official OIV method uses specific gravity for wine alcohol determination [167]. Instrumental methods such as HPLC and GC may also be used [169]. Fletcher and van Staden [170] described an automated sequential injection analysis technique utilising dichromate oxidation and spectrophotometric detection for the determination of ethanol in distilled liquors. Recently, the suitability of rapid, multi-component non-specific NIR spectroscopic methods for alcohol has been demonstrated. These methods rely on extensive calibration protocols to ensure accuracy [14,15,40].

Volatile acidity: Volatile acidity is defined as those wine acids which may readily be removed by steam distillation. Volatile acidity is an indicator of wine spoilage and is therefore regulated as a quality assurance parameter. Spoilage may result from bacterial action such as caused by acetic acid bacteria or spoilage yeasts, such as *Brettanomyces*. Since extrinsic factors may also play a role in development of volatile acids (for example in some dessert wines), specific legal limits are often dependent on the class or style of the wine [94,169]. The OIV prescribes steam distillation and titrimetry as the reference method for volatile acidity [167]. Enzymatic and flow injection methods as well as HPLC, GC and NIR spectroscopic methods have also been described for this purpose [14,15,168,169].

Sulphur dioxide: Sulphur dioxide is widely used in the wine industry as a chemical preservative and inhibitor of microbiological activity as well as an antioxidant to reduce chemical and enzymatic browning. Due to its negative sensory properties and adverse health effects, the sulphur dioxide content of wines is regulated. Sulphur dioxide can exist in inter-convertible free and bound states, the regulated levels of which vary depending on the

type and style of wine as well as between bulk and bottled wines [94,169]. The official OIV analysis method involves oxidation of separated sulphur dioxide followed by titremetry. Free and total sulphur dioxide are separately determined in this way by entrainment at low temperature and high temperature, respectively. Titration with iodine may be used as a rapid alternative method, although this procedure is known to be inaccurate. Instrumental methods described for sulphur dioxide analysis include flow injection analysis, enzymatic analysis, HPLC, GC, potentiometry and polarography, UV and Vis spectrophotometry, atomic absorption and fluorometric spectrometry as well as NIR spectroscopic methods [14,169].

Reducing sugars: The principal sugars utilised by yeast in alcoholic fermentation are glucose and fructose, referred to as reducing sugars as they are capable of reducing copper (as Cu II), a characteristic which is used in their analysis. The reducing sugar content is an important regulatory parameter that is used to classify wine styles [94]. Analytically, reducing sugars may be determined by chemical, enzymatic, flow injection analysis and HPLC [97] techniques, while GC may also be utilised following derivatisation [168,171]. Spectroscopic methods, such as NIR, have also been described for the determination of reducing sugars [14,15]. The official OIV method is based on the reduction of Cu II in boiling alkaline medium and determination of the remaining copper. In wine styles where the addition of sugar (usually sucrose) to the finished product is allowed, such as sparkling wines, these are subjected to a preliminary acid hydrolysis to convert disaccharides to their component reducing sugars [167]. Chaptalisation (pre-fermentation addition of sugar) is typically illegal in warmer growing conditions such as encountered in Africa, where grapes usually develop adequate sugar levels. The addition of sucrose to the must can only be detected by stable isotope analysis since complete hydrolysis of sucrose at normal wine pH levels is expected in the finished product [94].

Heavy metals: Many minerals are found in wine and in most instances these reflect uptake characteristics of the rootstock and climatic influences on the rate of transpiration. Since heavy metals typically precipitate during fermentation, their elevated occurrence in finished wine is usually associated with contamination after fermentation [94]. Heavy metals are determined in wine by spectrophotometric and spectroscopic techniques. Due to the low maximum levels that are typically enforced for toxic elements, specialised techniques such as graphite furnace atomic absorption spectroscopy (AAS) (for Pb and Cd) and hydride generation AAS (for As and Hg) are prescribed by the OIV. Flame AAS methods are used for elements such as copper, iron and tin, for which relatively high maximum levels are typically enforced [167]. Onianwa *et al.* [43] successfully applied flame AAS for the

determination of various metals, including lead and cadmium, in non-alcoholic wines using suitable sample mineralisation and pre-concentration techniques. Inductively coupled plasma emission spectroscopy (using both optical and mass spectrometric detection) may also be used for multi-element analysis. Dessuy *et al.* [44] developed and validated a method for the determination of lead in wine using electrothermal AAS. The use of various chemical modifiers was investigated and palladium was found to produce optimal stabilisation of lead during pyrolysis. The optimised procedure enabled the determination of lead in wine without any sample preparation with a limit of detection of 0.5 µg/L. Since this procedure is fully automated and sufficiently sensitive, it is suited for routine regulatory determination of lead in wines.

Preservatives: Antimicrobial agents are used to confer microbial stability to wine, the most frequently used being sulphur dioxide. Other preservatives such as sorbic acid, benzoic acid, dimethyl dicarbonate and natamycin are also allowed. Of these preservatives only sulphur dioxide and dimethyl dicarbonate possess reasonable wide-spectrum antimicrobial properties, while natamycin is prohibited in some countries (notably the EU). Dimethyl dicarbonate can effectively sterilise wine if used just before bottling. This compound decomposes rapidly to carbon dioxide and methanol and therefore produces no sensory defect or residue. However, it has low solubility and is corrosive and therefore requires expensive equipment for effective application. Sorbic acid and benzoic acid (or their sodium salts) generally have low effectiveness and produce negative sensory effects at higher concentrations. Their use is therefore subject to legislated maximum permitted concentrations [94]. Sorbic acid and benzoic acid may be determined by spectrophotometry, but are more often analysed in wine by HPLC with UV/Vis detection [169]. For example, at the Department of Agriculture, Forestry and Fisheries in South Africa, sorbic acid in wine is determined by direct injection RP-LC-UV utilising ion pairing or an acidic mobile phase to optimise chromatographic efficiency. Ultraviolet detection at ~260 nm confers sufficient selectivity to the technique to yield detection limits in the low mg/L range. Natamycin at its effective concentrations may also be determined in wine using HPLC with UV/Vis detection, but for demonstration of compliance with EU standards, more sensitive methodologies are required. Alberts *et al.* [172] recently described a simple, robust and fast LC-ESI-MS/MS method for the determination of natamycin in wine. Sample preparation involved dilution followed by direct elution from aminopropyl SPE cartridges. The application of mutually supporting sample pre-treatment and chromatographic separations to eliminate matrix-related ion suppression enabled quantitative determination of natamycin in wine with external standard calibration. This critical benefit rendered the method suitable for routine

analysis of large numbers of samples in support of the wine export industry to the EU. The method complied with EU standards in terms of sensitivity and selectivity for this application and was also used to study the degradation kinetics of natamycin in the wine matrix (an important aspect from a regulatory point of view).

Methanol: Methanol is usually present in wine in relatively small quantities and never accumulates to toxic levels when using legitimate winemaking procedures. In humans methanol is oxidised to formaldehyde and formic acid, both of which are toxic to the central nervous system. As methanol is derived from the pectin content of the fermentable substrate, red wines typically evolve more methanol than white wines, and pectolytic enzymes added to the juice or wine to aid clarification may further increase methanol concentrations. The addition of distilled spirits to wine, such as in fortified wines, may also affect the methanol content [94,169]. Wine methanol content is therefore typically regulated by legislation. Methanol is usually determined by GC-FID following quantitative distillation of the wine to eliminate non-volatile constituents.

Wine authenticity: Establishing conformity with laws and regulations governing the wine industry is often dependent on the development of sophisticated analysis techniques. Because of the wide range of possible adulteration practices and the complexity of wine, these methods are often specifically designed for each type of adulteration [94]. For example, de Villiers *et al*. [165] developed HPLC and CE methods for the analysis of the artificial dyes brilliant blue and azorubine in red wines. Liquid–liquid extraction followed by ion-pair LC analysis allowed separation of these dyes from wine polyphenols to achieve detection limits in the parts per billion range with reliable UV-spectral identification. On the other hand CE analysis following SPE sample clean-up provided higher efficiency, reduced solvent consumption and faster analyses for the same analyses [165].

4.4.2. Food safety

Pesticides: Synthetic organic pesticides are used for vineyard disease, pest and weed control. Integrated pest management schemes aim to limit the application of these treatments while increasing their effectiveness through the application of combined expertise in the fields of plant pathology, economic entomology, plant nutrition, weed control and soil science. However, programs to monitor wines for the presence of these substances are required for consumer health protection [94].

Traditional pesticide residue analytical methods employ LLE or SPE for sample preparation prior to GC or HPLC analysis. Sandra *et al.* [173] described a multi-residue GC-MS method based on SBSE for the analysis of pesticide residues in aqueous foods and grapes. Solid samples were pre-extracted with methanol, diluted and extracted by SBSE followed by automated sample introduction by thermal desorption. Pesticide residues were identified using retention time locked GC-MS operated in full scan mode. Quantitation was performed using standard addition or isotope dilution, since matrix interferences affected analyte recoveries. The technique yielded detection limits in the sub-ppb range for over 350 pesticides [173]. The same approach was also used for the determination of dicarboximide fungicides in white wines, and provided detection limits in the low to sub-µg/L range. For thermolabile congeners, the accuracy of the technique was verified by SBSE followed by liquid desorption and analysis with LC-APCI-MS [86]. The quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction method is a novel sample extraction technique used effectively in multi-residue methods in combination with MS analysis techniques. Afify *et al.* [174] validated a method for the determination of 150 pesticide residues in grapes using QuEChERS in combination with LC-ESI-MS in the positive ionisation mode. Ionisation suppression effects were compensated with matrix matched calibration standards and recoveries of target pesticides were 70-110%.

Toxins and mycotoxins: Ethyl carbamate is a carcinogen that is universally present in wines where it is mainly formed by the acid-catalysed reaction between ethanol and urea. Its concentration in liquor products is therefore regulated in several countries. Ethyl carbamate accumulates in wine over time and is also present in distilled products such as brandy. Since the presence and associated health risks of ethyl carbamate became known, measures have been instigated to reduce the risk of contamination, for example limiting vineyard nitrogen fertilisation to reduce the formation of precursors (such as urea) [94,175].

Ethyl carbamate is usually determined by GC following SPE or LLE sample clean-up and pre-concentration. Waldner and Augustyn [176] used GC-MS to conduct a survey of the levels of ethyl carbamate in South African wines. Although red wines were found to accumulate more ethyl carbamate compared to white wines, low concentrations were mostly found. Alberts *et al.* [175] recently reported a novel RP SPE- normal phase LC-APCI-MS/MS method for the determination of ethyl carbamate in liquor products. This method offered good sensitivity and selectivity and its applicability for the analysis of wines, fortified wines and distilled spirits such as brandy was demonstrated. The authors reported levels of ethyl

carbamate in South African products, as well as the factors responsible for its formation. Despite a clear correlation with age, all products contained low concentrations.

Ochratoxin A is a carcinogenic mycotoxin produced by several fungi. Since grapes are frequently contaminated, this compound also occurs in wine. Ochratoxin A is determined by RP-LC with FLD or MS detection. Confirmation of Ochratoxin A utilising HPLC-FLD has been achieved by derivatisation to the corresponding methyl ester products, while MS detection inherently lends itself to structurally specific confirmation. The low levels of occurrence of ochratoxin A in wine generally necessitate sample pre-concentration, which is most often performed using immunoaffinity columns.

Aboul-Enein *et al.* [177] described a LLE sample preparation procedure utilising chloroform for the analysis of ochratoxin A in wine. Various surveys of the ochratoxin A content of South African wines found concentrations that were well below the suggested EU regulatory limit [178,179]. A study of the occurrence of ochratoxin A and identification of octratoxigenic microbiota in Tunisian vineyards found that *Aspergillus carbonarius* is the principal cause of contamination in Tunisian grapes. The potential for ochratoxin A contamination is highly variable and increases during grape ripening, while some grape varieties are more susceptible [180]. Selouane *et al.* [181] studied the effect of temperature, water activity and incubation time on growth and ochratoxin A production by fungi isolated from Moroccan grapes. Morocco has a warm, humid climate which is conducive to the development and growth of molds, and therefore the risk of mycotoxin contamination is relatively high. A review of the occurrence and legislation of mycotoxins in food and feed from Morocco reported relatively high levels of ochratoxin A in wines (red wines were particularly affected) [182].

4.5. Conclusions

This survey of the analysis of grape, wine and derived beverages performed in Africa allows several general conclusions to be drawn. Clearly, based on the number of references reported herein, extensive and increasing analytical research involving these products is performed on the African continent. The significant increase in the number of papers from African authors dealing with this topic, especially during the last decade, is evident from Figure 4.7, which presents a concise summary of the reports cited in this review. It should be noted that much of the regulatory analyses, often performed using instrumental techniques, are rarely reported in the scientific literature, and therefore the amount of research

performed on this topic arguably significantly exceeds an estimate based solely on the papers cited here.

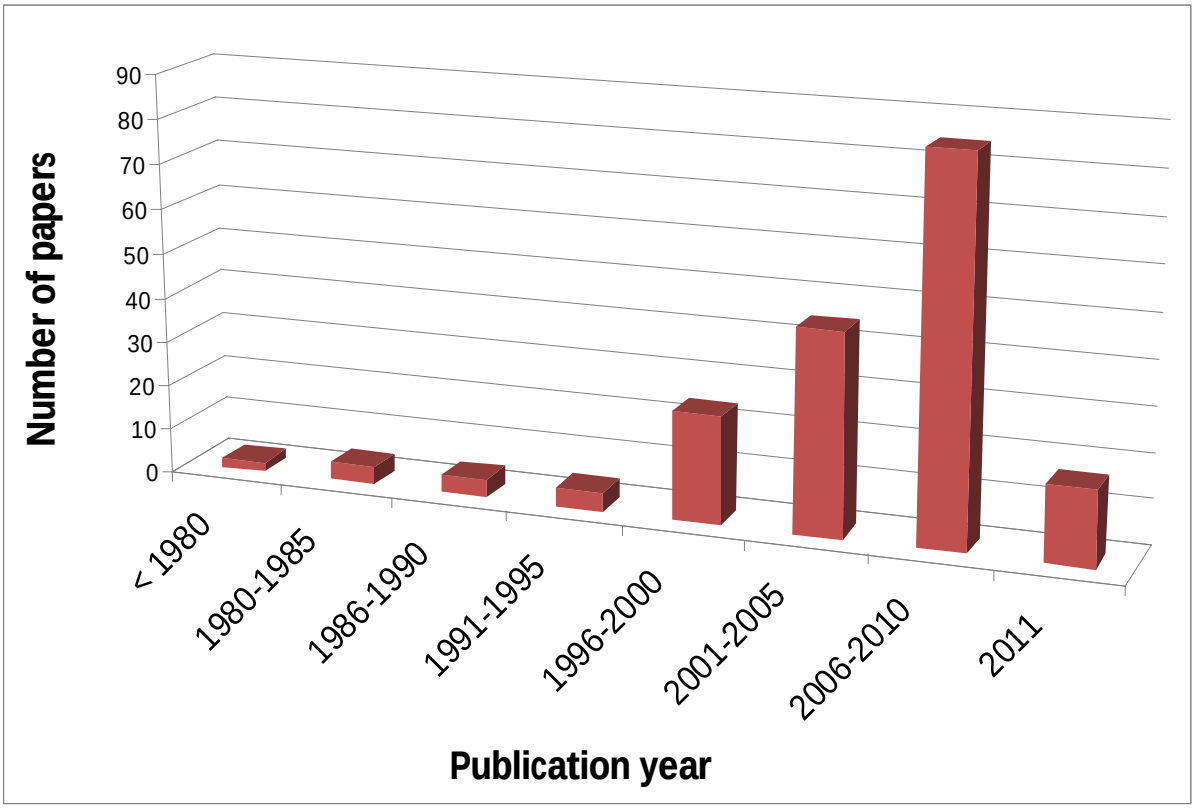


Figure 4.7. Summary of the number of references in scientific literature dealing with the analysis of wine, where one or more authors are from an African institution.

In terms of the analytical methods employed in these studies, since the 1970's, there has been a continuous trend in using more advanced analytical instrumentation to shed light on the chemical composition of these samples. This phenomenon may be ascribed first of all to concomitant developments in methods of instrumental analysis. Secondly, accurate analytical data play an important role in many spheres of research involving grapes and their derived products. As research questions become more detailed and challenging, more advanced chemical analysis methods are therefore required. Table 4.1 provides an overview of the most important milestones in analytical methods applied to wine analysis in the African context.

Table 4.1. Summary of the most important milestones in instrumental chemical analytical methods applied to wine analysis in the African context.

Milestones	Selected references
Spectroscopy	
Quantitation of grape and wine compounds Establishment of PLS-based algorithms for the quantitation of important grape and wine quality parameters using NIR, FT-NIR and FT-MIR allows high-throughput quantitation of wine constituents.	[26,27,32,38]
Application in yeast breeding and identification studies High-throughput screening of hybrid yeasts based on their fermentation profiles obtained by FT-MIR spectroscopy and chemometric techniques as well as identification of pure cultures of the spoilage yeast *Brettanomyces bruxellensis.*	[40,41]
Authentication studies FT-MIR spectra of 5 important single cultivar wines were used to discriminate between cultivars.	[42]
Gas phase separations	
Capillary GC columns The introduction of capillary GC columns resulted in a dramatic increase in chromatographic resolution and sensitivity compared to packed columns. Nowadays almost exclusively used for wine analysis.	[42,56,57,81]
Gas chromatography – mass spectrometry (GC-MS) Coupling of MS with GC separation allows on-line identification of unknowns based on their mass spectra. MS also offers enhanced sensitivity, especially when operated in selected ion monitoring (SIM) mode.	[52,53,64,81,93]
Gas chromatography – olfactometry (GC-O) GC-O combines the separation power of GC with the selectivity and sensitivity of the human nose to study odour-active compounds in wine.	[82]
Solid phase extraction (SPE) in combination with GC SPE is a selective sample preparation procedure which enables targeting of specific chemical classes by removal of interfering wine constituents and pre-concentration prior to GC analysis.	[81,89]
Solid phase micro-extraction (SPME) in combination with GC SPME is a solventless and sensitive sorptive sample preparation technique for GC which is used extensively in wine analysis; a wide selection of phases is available to tune selectivity	[64,79,80,83,84]
Stir bar sorptive extraction (SBSE) in combination with GC Another sorptive extraction method, SBSE offers increased sensitivity compared to SPME due to larger amount of sorptive phase (PDMS); has been used as alternative to SPME for wine analysis	[58,60,83,87]

Comprehensive 2-dimensional gas chromatography (GC´GC)	[79,80,83]
GC´ GC provides drastic improvement of chromatographic resolution and sensitivity due to the use of 2 orthogonal separation mechanisms; only recently been applied to wine.	
Liquid phase separation	
High performance liquid chromatography (HPLC)	[100,101,104,110,112,114]
The application of automated high pressure instrumentation and columns has significantly improved the routine quantitative analysis of non-volatiles in the wine industry.	
Liquid chromatography-mass spectrometry (LC-MS)	[86,111,138-149,152,174]
MS is a powerful structural elucidation tool and sensitive detector when used in combination with HPLC separation, which has found extensive application in wine analysis in recent years	
Liquid chromatography tandem mass spectrometry (LC-MS^n)	[131,158-160,172,175]
Tandem mass spectrometric techniques provide improved sensitivity and selectivity as well as improved structural elucidation performance, especially relevant for trace-level wine constituents.	
Capillary electrophoresis (CE) and CE-MS	[96,164,165]
CE provides several potential benefits compared to HPLC, primarily improved separation efficiency, although the technique has found limited application in wine analysis in Africa.	
Ultra high pressure liquid chromatography (UHPLC)	[103,130-133,160,162]
A recent development in HPLC, where small particle-packed columns are operated at elevated pressures (> 400 bar) for improved speed or efficiency; recently finding increasing application in wine analysis.	
High temperature liquid chromatography (HTLC)	[103,130-133]
Elevated temperature is used primarily to provide shorter analysis times, often in combination with UHPLC; limited application to wine analysis to date.	

The past few years have seen a significant increase in the application of spectroscopic techniques in combination with multivariate data analysis methods for especially wine analysis. The papers cited show that IR spectroscopy has huge potential for rapid low-cost quantitative and qualitative applications throughout the production chain of winemaking. It is foreseen that some of the new global trends in viticulture, particularly the use of remote sensing and portable spectrometers, will be increasingly used in monitoring grape quality in vineyards in Africa. The continent has substantial local expertise, as well as international collaborations, to exploit IR technology for the purpose of sustainability in agricultural production. In terms of product authentication, spectroscopic techniques, including NMR spectroscopy, which has to date seen little application in grape and wine analysis in Africa, will without doubt also be increasingly used in the future. Finally, the emerging technologies of IR and NMR imaging that frequently combines microspectrometry for *in situ* visualisation

of chemical features in whole tissue, will feature more prominently in addressing more fundamental research problems of biological nature.

Also clear from the papers cited in this review, is the growing use of advanced chromatographic methods for wine and grape analysis. In terms of gas-phase separations, GC-FID remains a popular method for routine analysis of volatiles, although GC-MS is increasingly often being used both for quantitative and qualitative analyses in this field. This development may be linked to the obvious benefits of the technique in terms of sensitivity and identification power, as well as the fact that bench-top GC-MS instruments have become relatively affordable. Other important fields in gas-phase separations include sample preparation, alternative, more sensitive and selective detection strategies such as TOF-MS and tandem MS, and in recent years the application of GC×GC to wine analysis.

An overview of the application of HPLC for wine analysis in Africa highlights the importance of this technique, especially for purposes of routine analysis of a large number of non-volatile compounds. Data generated in this manner has been used broadly in studies involving wine chemistry and relating various manufacturing processes to wine chemical composition. In addition to the extensive use of HPLC for routine analyses, the technique has made a significant contribution to the detailed investigation of the complex chemistry of grapes and especially wine. For this type of research, LC-MS is increasingly being used for identification purposes of novel compounds (often in combination with preparative isolation and NMR) [183]. Moreover, a recent trend, also evident from the research performed in Africa, is the application of tandem mass spectrometry for the selective detection of trace-level compounds. Capillary electrophoresis shows promise for analysis of specific compounds where this technique provides benefits compared to HPLC. However, the relative complexity of the technique and limited availability of instrumentation and expertise in Africa means that HPLC will remain the chromatographic method of choice for the analysis of non-volatile constituents in wine.

Finally, the use of advanced (often multivariate) statistical methods in combination with analytical data for wine and grapes has developed significantly in the last decade [22]. This may partially be linked to the developments in analytical methodologies, as the increasing amount of information obtained using advanced spectroscopic and chromatographic techniques has highlighted the importance of extracting the information relevant for a particular experiment. Related to this is an increasing trend in the application of statistical analytical methods for the unsupervised analysis of grapes and wine. In these instances, the

goal is not target analysis of a selected number of compounds, but rather to find differences between samples based on certain parameters, where the compounds differing are not known *a priori*. With the amount of information that may be obtained in a single analysis continuously increasing, this trend is expected to become more important in the future.

It should be noted that much of the research performed on grapes and wine is of an inherent inter-disciplinary nature [62]. Analytical techniques are extensively employed in various research areas related to the production of grapes and wine, including viticulture, soil science, horticulture, microbiology, biotechnology, etc. This aspect has also served to drive developments in the field of wine and grape analysis. Therefore, a large part of the wine research performed on the continent critically hinges on analytical techniques to obtain quantitative data for a wide range of compounds.

Clearly, analytical chemistry plays an important part in ongoing research aimed at improved understanding of wine production, with the ultimate goal of producing better products. In view of the chemical complexity of grapes and their derived products, these trends are expected to increase further in future, and in this manner analytical methods will continue to play an influential role in the understanding of the chemical composition of grapes, wine and their derived products on the African continent.

Acknowledgements

Financial support was provided by the South African National Research Foundation (NRF) and Winetech.

References

[1] J. Robinson, The Oxford Companion to Wine, Oxford University Press, Oxford, U.K. (1994).
[2] C. Theron, Winelands of the World, African Sun Media, Stellenbosch, South Africa (2005).
[3] South African wine industry information and systems, http://www.sawis.co.za/info/statistics.php, cited 18 August 2011.
[4] Organisation Internationale de la Vigne et du Vin (OIV), Paris, France, http://www.oiv.int., cited 29 August 2011.
[5] G. Grindlay, J. Mora, L. Gras, M.T.C. de Loos-Vollebregt, Anal. Chim. Acta 691 (2010) 18.
[6] I.J. Kosir, M. Kocjancic, N. Ogrinc, J. Kidric, Anal. Chim. Acta 429 (2001) 195.
[7] M. Ruiz-Altisent, L. Ruiz-Garcia, G.P. Moreda, R. Lu, N. Hernandez-Sanchez, E.C. Correa, B. Diezma, B. Nicolaï, J. García-Ramos, Comp. Electr. Agric. 74 (2010) 176.
[8] Z.G. Cerovic, N. Moise, G. Agati, G. Latouche, N. Ben Ghozlen, S. Meyer, J. Food Compos. Anal. 21 (2008) 650.
[9] L. Hartmanova, V. Ranc, B. Papouskova, P. Bednar, V. Havlicek, K. Lemr, J. Chromatogr. A 1217 (2010) 4223.
[10] C.M. McGoverin, J. Weeranantanaphan, G. Downey, M. Manley, Journal of Near Infrared Spectroscopy 18 (2010) 87.
[11] A. Versari, G.P. Parpinello, A.U. Mattioli, S. Afr. J. Enol. Vitic. 28 (2007) 6.
[12] A. Versari, R.B. Boulton, G.P. Parpinello, Food Chem. 106 (2008) 397.
[13] W.J. du Toit, J. Lisjak, J. Marais, M. du Toit, S. Afr. J. Enol. Vitic. 27 (2006) 57.
[14] C.D. Patz, A. Blieke, R. Ristow, H. Dietrich, Anal. Chim. Acta 513 (2004) 81.
[15] R. Bauer, H. Nieuwoudt, F.F. Bauer, J. Kossmann, K.R. Koch, K.H. Esbensen, Anal. Chem. 80 (2008) 1371.
[16] D. Cozzolino, R.G. Dambergs, L. Janik, C. Wu, M. Gishen, Near Infr. Spectr. 14 (2009) 279.
[17] M. Manley, G. Downey, V. Beaten, Spectroscopic technique: Near-infrared (NIR) spectroscopy, modern techniques for food authentication, D.-W. Sun (Ed), Elsevier, Amsterdam, The Netherlands (2008), p. 65.
[18] M. Manley, G. Downey, V. Beaten, Spectroscopic technique: Fourier transform near-infrared (FT-NIR) spectroscopy, modern techniques for food authentication, D.-W. Sun (Ed), Elsevier, Amsterdam, The Netherlands (2008), p. 117
[19] L. Laghi, G.P. Parpinello, D.D. Rio, L. Calani, A.U. Mattioli, A. Versari, Food Chem. 121 (2010) 783.
[20] T. Næs, T. Isaksson, T. Fearn, T. Davies, A User-friendly guide to multivariate calibration and classification, NIR Publications, Chichester, UK (2002).
[21] R. Leardi, Chemometric methods in food authentication, modern techniques for food authentication, D.-W. Sun (Ed), Elsevier, Amsterdam, The Netherlands (2008).
[22] S.O. Paul, Chemometrics Intel. Lab. Sys. 97 (2009) 104.
[23] T. Kourti, Ann. Rev. Control 27 (2003) 131.
[24] T. Kourti, Multivariate statistical process control and process control, using latent variables, Comprehensive chemometrics, S.D. Brown, R. Tauler, B. Walczak (Ed's), Elsevier, Amsterdam, The Netherlands (2009), p. 21.
[25] M. Gishen, M. Holdstock, I.S. Pretorius, M. Manley, F.F. Bauer, Austr. Grapegrower Winemaker (2000) 75.
[26] H.H. Nieuwoudt, B.A. Prior, I.S. Pretorius, M. Manley, F.F. Bauer, J. Agric. Food Chem. 52 (2004) 3726.
[27] M. Swanepoel, M. du Toit, H.H. Nieuwoudt, S. Afr. J. Enol. Vitic. 28 (2007) 140.
[28] Near infrared technology in the agricultural and food industries, P. C. Williams, K. H. Norris (Ed's), American Association of Cereal Chemists, St. Paul, U.S.A. (2001).
[29] B.M. Nicolai, K. Beullens, E. Bobelyn, A. Peirs, W. Sayes, K. Theron, J. Lammertyn, Post Harvest Biol. Techn. 46 (2007) 99.

[30] H.H. Eriksson, E. Johansson, N. Kettaneh-Wold, S. Wold, Multi- and megavariate data analysis – principles and applications, Umetrics AB. (1999).
[31] W.F. McClure, Near infrared technology in the agricultural and food industries, P. C. Williams, K. H. Norris (Ed's), American Association of Cereal Chemists, St. Paul, U.S.A. (2001), p.109.
[32] G. Baumgarten, S. Afr. J. Enol. Vitic. 8 (1987) 76.
[33] D.L. Wetzel, Contemporary near-infrared instrumentation, Near infrared technology in the agricultural and food industries, P. C. Williams, K. H. Norris (Ed's), American Association of Cereal Chemists, St. Paul, U.S.A. (2001), p.129.
[34] D. Cozzolino, W. Cynkar, N. Shah, P. Smith, Food Res. Int. 44 (2011) 181.
[35] WineScan FT 120 Type 77110 and 77310 Reference manual, Issue 4 GB, August 2000, p 35.
[36] http://www.foss.dk, cited 18 August 2011
[37] http://www.afrodata.org, cited 18 August 2011
[38] M. Manley, A. Van Zyl, E.E.H. Wolf, S. Afr. J. Enol. Vitic. 2 (2001) 93.
[39] J. Pink, M. Naczk, D. Pink, J. Agric. Food Chem. 46 (1998) 3667.
[40] H.H. Nieuwoudt, I.S. Pretorius, F.F. Bauer, D.G. Nel, B.A. Prior, J. Microbiol. Meth. 67 (2006) 248.
[41] A. Oelofse, S. Malherbe, I.S. Pretorius, M. du Toit, Int. J. Food Microbiol. 143 (2009) 136.
[42] L. Louw, K. Roux, A. Tredoux, O. Tomic, T. Naes, H.H. Nieuwoudt, P. van Rensburg, J. Agric. Food Chem. 57 (2009) 2623.
[43] P.C. Onianwa, I.G. Adetola, C.M.A. Iwegbue, M.F. Ojo, O.O. Tella, Food Chem. 66 (1999) 275.
[44] M.B. Dessuy, M.G.R. Vale, A.S. Souza, S.L.C. Ferreira, B. Welz, D.A. Katskov, Talanta 74 (2008) 1321.
[45] P.P. Coetzee, F. Vanhaecke, Anal. Bioanal. Chem. 383 (2005) 977.
[46] P.P. Coetzee, F.E. Steffens, R.J. Eiselen, O.P. Augustyn, L. Balcaen, F. Vanhaecke, J. Agric. Food Chem. 53 (2005) 5060.
[47] G. van der Linde, J.L. Fischer, P.P. Coetzee, S. Afr. J. Enol. Vitic. 31 (2010) 143.
[48] C. Vorster, L. Greeff, P.P. Coetzee, S. Afr. J. Chem. 63 (2010) 207.
[49] C.J. van Wyk, O.P.H. Augustyn, P. de Wet, W.A. Joubert, Am. J. Enol. Vitic. 30 (1979) 167.
[50] A.C. Houtman, J. Marais, C.S. du Plessis, S. Afr. J. Enol. Vitic. 1 (1980) 27.
[51] J. Marais, P.C. van Rooyen, C.S. Du Plessis, S. Afr. J. Enol. Vitic. 2 (1981) 19.
[52] A. Smit, R.R. Cordero Otero, M.G. Lambrechts, I.S. Pretorius, P. van Rensburg, Appl. Environ. Microbiol. 51 (2003) 4909.
[53] G. Versini, A. Rapp, J. Marais, F. Mattivi, M. Spraul, Vitis 35 (1996) 15.
[54] C. Knoll, M. du Toit, S. Schnell, D. Rauhut, S. Irmler, Appl. Microbiol. Biotechn. 89 (2011) 1051.
[55] R.P. Tracey, T.J. Britz, Appl. Environ. Microbiol. 55 (1989) 1617.
[56] O.P.H. Augustyn, S. Afr. J. Enol. Vitic. 3 (1982) 37.
[57] L. Louw, A.G.J. Tredoux, P. van Rensburg, M. Kidd, T. Naes, H.H. Nieuwoudt, S. Afr. J. Enol. Vitic. 31 (2010) 213.
[58] A. Tredoux, A. de Villiers, P. Majek, F. Lynen, A. Crouch, P. Sandra, J. Agric. Food Chem. 56 (2008) 4286.
[59] B.T. Weldegergis, A. de Villiers, A.M. Crouch, Food Chem. 128 (2011) 1100.
[60] B.T. Weldegergis, A.M. Crouch, J. Agric. Food Chem. 56 (2008) 10225.
[61] D. Rossouw, F.F. Bauer, S. Afr. J. Enol. Vitic. 30 (2009) 101.
[62] J.P. Moore, B. Divol, P.R. Young, H.H. Nieuwoudt, V. Ramburan, M. du Toit, F.F. Bauer, M.A. Vivier, Biotechn. J. 3 (2008) 1355.
[63] I.S. Pretorius, Yeast 16 (2000) 675.
[64] J.H. Swiegers, R. Willmott, A. Hill-Ling, D.L. Capone, K.H. Pardon, G.M. Elsey, K.S. Howell, M.A. de Barros Lopes, M.A. Sefton, M. Lilly, I.S. Pretorius, Modulation of

volatile thiol and ester aromas by modified wine yeast, Developments in food science 43, Flavour Science Recent Advances and Trends, W. Bredie, M. Petersen (Ed's), Elsevier, Amsterdam, The Netherlands (2006).
[65] C. Louw, D. la Grange, I.S. Pretorius, P. van Rensburg, J. Biotechn. 125 (2006) 447.
[66] M. Lilly, F.F. Bauer, M.G. Lambrechts, J.H. Swiegers, D. Cozzolino, I.S. Pretorius, Yeast 23 (2006) 641.
[67] M. Lilly, M.G. Lambrechts, I.S. Pretorius, Appl. Environm. Microbiol. 66 (2000) 744.
[68] M. Lilly, F.F. Bauer, G. Styger, M.G. Lambrechts, I.S. Pretorius, FEMS Yeast Res. 6 (2006) 726.
[69] C.L.C. Steger, M.G. Lambrechts, J. Ind. Microbiol. Biotechn. 24 (2000) 431.
[70] N.P. Jolly, B.J.H. Janse, T.J. van Rooyen, J.H. Louw, Am. J. Enol. Vitic. 44 (1993) 217.
[71] D. Rossouw, T. Naes, F. Bauer, BMC Genomics 9 (2008) 530.
[72] A.J.J. Zietsman, D. de Klerk, P. van Rensburg, FEMS Yeast Res. 11 (2011) 88.
[73] R. Bauer, M. du Toit, J. Kossmann, Int. J. Food Microbiol. 137 (2010) 28.
[74] R. Bauer, D.A. Cowan, A. Crouch, J. Agric. Food Chem. 58 (2010) 3243.
[75] B.V. Burger, B. Marx, M. le Roux, W.J.G. Burger, J. Chromatogr. A 1121 (2006) 259.
[76] F.P. van Jaarsveld, S. Hattingh, P. Minnaar, M. Blom, S. Afr. J. Enol. Vitic. 30 (2009) 1.
[77] F.P. van Jaarsveld, S. Hattingh, P. Minnaar, S. Afr. J. Enol. Vitic. 30 (2009) 16.
[78] F.P. van Jaarsveld, S. Hattingh, P. Minnaar, S. Afr. J. Enol. Vitic. 30 (2009) 24.
[79] B.T. Weldegergis, A.M. Crouch, T. Górecki, A. de Villiers, Anal. Chim. Acta 701 (2011) 98.
[80] B.T. Weldegergis, A. de Villiers, C. McNeish, S. Seethapathy, A. Mostafa, T. Górecki, A.M. Crouch, Food Chem. 129 (2011) 188.
[81] I. Souid, Z. Hassene, E.V.A.S. Palomo, M.S. Perez-Coello, A. Ghorbel, J. Food Qual. 30 (2007) 718.
[82] B.V. Burger, Z. Munro, J. Chromatogr. A 370 (1986) 449.
[83] J. Vestner, S. Malherbe, M. du Toit, H.H. Nieuwoudt, A. Mostafa, T. Górecki, A.G.J. Tredoux, A. de Villiers, J. Agric. Food Chem. (2011) DOI: 10.1021/jf2028208.
[84] W.A. Wan Ibrahim, H. Farhani, M.M. Sanagi, H.Y. Aboul-Enein, J. Chromatogr. A 1217 (2010) 4890.
[85] E. Baltussen, P. Sandra, F. David, C. Cramers, J. Microcol. Sep. 11 (1999) 737.
[86] P. Sandra, B. Tienpont, J. Vercammen, A. Tredoux, T. Sandra, F. David, J. Chromatogr. A 928 (2001) 117.
[87] B.T. Weldegergis, A.G.J. Tredoux, A.M. Crouch, J. Agric. Food Chem. 55 (2007) 8696.
[88] S. Malherbe, V. Watts, H.H. Nieuwoudt, F.F. Bauer, M. du Toit, J. Agric. Food Chem. 57 (2009) 5161.
[89] E. Campo, J. Cacho, V. Ferreira, J. Chromatogr. A 1140 (2007) 180.
[90] O.P.H. Augustyn, S. Afr. J. Enol. Vitic. 10 (1989) 8.
[91] D. Rossouw, R. Olivares-Hernandes, J. Nielsen, F.F. Bauer, Appl. Environ. Microbiol. 75 (2009) 6600.
[92] J. Grimplet, M.D. Wheatley, H.B. Jouira, L.G. Deluc, G.R. Cramer, J.C. Cushman, Proteomics 9 (2009) 2503.
[93] H.S.M. Ali, R. Patzold, H. Bruckner, Amino Acids 38 (2010) 951.
[94] R.S. Jackson, Wine Science, Principles, Practice, Perception, Academic Press, New York, U.S.A. (2000).
[95] A.L. Waterhouse, S.E. Ebeler, Chemistry of wine flavor, Proceedings of a symposium at the 213th national meeting of the American Chemical Society, held 13-17 April 1997, in San Francisco, California, U.S.A. (1998).
[96] A. de Villiers, F. Lynen, A. Crouch, P. Sandra, Eur. Food Res. Techn. 217 (2003) 535.
[97] A. de Villiers, F. Lynen, A. Crouch, P. Sandra, Chromatographia 59 (2004) 403.

[98] A. de Villiers, T. Gorecki, F. Lynen, R. Szucs, P. Sandra, J. Chromatogr. A 1161 (2007) 183.
[99] A.Y. Smit, W.J. du Toit, M. du Toit, S. Afr. J. Enol. Vitic. 29 (2008) 109.
[100] J.D. Cilliers, C.J. Van Wyk, S. Afr. J. Enol. Vitic. 6 (1985) 35.
[101] J.G. Lashbrooke, P.R. Young, A.E. Strever, C. Stander, M.A. Vivier, Austr. J. Grape Wine Res. 16 (2010) 349.
[102] G.O. Armstrong, M.G. Lambrechts, E.P.G. Mansvelt, D.P. van Velden, I.S. Pretorius, S. Afr. J. Sci. 97 (2001) 279.
[103] K.M. Kalili, A. de Villiers, J. Sep. Sci. 34 (2011) 854.
[104] D. de Beer, J.F. Harbertson, P.A. Kilmartin, V. Roginsky, T. Barsukova, D.O. Adams, A.L. Waterhouse, Am. J. Enol. Vitic. 55 (2004) 389.
[105] D.M. Goldberg, A. Karumanchiri, G.J. Soleas, E. Tsang, Am. J. Enol. Vitic. 49 (1998) 142.
[106] D.M. Goldberg, A. Karumanchiri, G.J. Soleas, E. Tsang, Am. J. Enol. Vitic. 50 (1999) 185.
[107] S.M. Basha, M. Musingo, V.S. Colova, Afr. J. Biotechn. 3 (2004) 523.
[108] M. Rossouw, J. Marais, S. Afr. J. Enol. Vitic. 25 (2004) 94.
[109] A.L. Waterhouse, S.F. Price, J.D. McCord, P. Lester, Reversed-phase high-performance liquid chromatography methods for analysis of wine polyphenols, Methods in enzymology, J.N. Abelson, M.I. Simon, H. Sies (Ed's), Academic Press, New York, U.S.A. (1999), p. 113.
[110] A. de Villiers, P. Majek, F. Lynen, A. Crouch, H. Lauer, P. Sandra, Eur. Food Res. Techn. 221 (2005) 520.
[111] A. de Villiers, G. Vanhoenacker, P. Majek, P. Sandra, J. Chromatogr. A 1054 (2004) 195.
[112] A. Liazid, G. Barbero, M. Palma, J. Brigui, C. Barroso, Chromatographia 72 (2010) 417.
[113] H.A. Guebailia, K. Chira, T. Richard, T. Mabrouk, A. Furiga, X. Vitrac, J.-P. Monti, J.-C. Delaunay, J.-M. Merillon, J. Agric. Food Chem. 54 (2006) 9559.
[114] H. Amira-Guebailia, J. Valls, T. Richard, X. Vitrac, J.P. Monti, J.C. Delaunay, J.M. Merillon, Food Chem. 113 (2009) 320.
[115] N.E. Es-Safi, S. Ghidouche, P.H. Ducrot, Molecules 12 (2007) 2228.
[116] A. El-Haitum, S.M. Amrani, N. Gougoulias, Z. Daoudi, L. Masheva, N. Mashev, Oxid. Commun. 31 (2008) 527.
[117] D. de Beer, E. Joubert, W.C.A. Gelderblom, M. Manley, S. Afr. J. Enol. Vitic. 23 (2002) 48.
[118] D. de Beer, E. Joubert, W.C.A. Gelderblom, M. Manley, J. Agric. Food Chem. 51 (2003) 902.
[119] D. de Beer, E. Joubert, W.C.A. Gelderblom, M. Manley, Food Chem. 90 (2005) 569.
[120] V. Roginsky, D. de Beer, J.F. Harbertson, P.A. Kilmartin, T. Barsukova, D.O. Adams, J. Sci. Food Agric. 86 (2006) 834.
[121] D. de Beer, E. Joubert, J. Marais, M. Manley, J. Agric. Food Chem. 54 (2006) 2897.
[122] Z. Peng, Y. Hayasaka, P.G. Iland, M. Sefton, P. Hoj, E.J. Waters, J. Agric. Food Chem. 49 (2000) 26.
[123] D. de Beer, E. Joubert, J. Marais, W. du Toit, B. Fourie, M. Manley, S. Afr. J. Enol. Vitic. 29 (2008) 39.
[124] D. de Beer, E. Joubert, J. Marais, M. Manley, S. Afr. J. Enol. Vitic. 29 (2008) 13.
[125] D. Youssef, H. El-Adawi, J. Appl. Sci. 6 (2006) 2944.
[126] M. Hmamouchi, N. Es-Safi, M. Lahrichi, A. Fruchier, E.M. Essassi, Am. J. Enol. Vitic. 47 (1996) 186.
[127] M. Hmamouchi, N. Es-Safi, E.M. Essassi, Fitoterapia 68 (1997) 332.
[128] A. Ghassempour, R. Heydari, Z. Talebpour, A.R. Fakhari, A. Rassouli, N. Davies, H.Y. Aboul-Enein, J. Liq. Chromatogr. Relat. Technol. 31 (2008) 2686.

[129] J.Y. Choi, S.J. Lee, S. Park, J.H. Lee, J.H. Shim, A.M. Abd El-Aty, J.S. Jin, E.D. Jeong, W.S. Lee, S.C. Shin, J. Sep. Sci. 33 (2010) 1192.
[130] A. de Villiers, D. Cabooter, F. Lynen, G. Desmet, P. Sandra, J. Chromatogr. A 1216 (2009) 3270.
[131] A. de Villiers, K.M. Kalili, M. Malan, J. Roodman, LCGC Europe 23 (2010) 466.
[132] A. de Villiers, K.M. Kalili, M. Malan, J. Roodman, LCGC North America 28 (2010) 978.
[133] A. de Villiers, D. Cabooter, F. Lynen, G. Desmet, P. Sandra, J. Chromatogr. A 1218 (2011) 4660.
[134] A. Oberholster, I.L. Francis, P.G. Iland, E.J. Waters, Austr. J. Grape Wine Res. 15 (2009) 59.
[135] R. Ristic, M.O. Downey, P.G. Iland, K. Bindon, I.L. Francis, M. Herderich, S.P. Robinson, Austr. J. Grape Wine Res. 13 (2007) 53.
[136] K. Bindon, P. Dry, B. Loveys, Austr. J. Grape Wine Res. 14 (2008) 91.
[137] N.E. Es-Safi, V. Cheynier, Flavanols and anthocyanins as potent compounds in the formation of new pigments during storage and aging of red wine, Red wine color, A.L. Waterhouse, J.A. Kennedy (Ed's), American Chemical Society, Washington, U.S.A. (2004), p. 143-159.
[138] N.E. Es-Safi, C. Le Guerneve, H. Fulcrand, V. Cheynier, M. Moutounet, Int. J. Food Sci. Techn. 35 (2000) 63.
[139] N.E. Es-Safi, C. Le Guerneve, H. Fulcrand, V. Cheynier, M. Moutounet, J. Agric. Food Chem. 47 (1999) 5211.
[140] N.E. Es-Safi, C. Le Guernevé, V. Cheynier, M. Moutounet, Magn. Res. Chem. 40 (2002) 693.
[141] N.E. Es-Safi, C. Le Guerneve, V. Cheynier, M. Moutounet, Tet. Lett. 41 (2000) 1917.
[142] N.E. Es-Safi, V. Cheynier, M. Moutounet, J. Food Compos. Anal. 16 (2003) 535.
[143] N.E. Es-Safi, H. Fulcrand, V. Cheynier, M. Moutounet, J. Agric. Food Chem. 47 (1999) 2088.
[144] N.E. Es-Safi, Food Chem. 88 (2004) 367.
[145] N.E. Es-Safi, C. Le Guerneve, V. Cheynier, M. Moutounet, J. Agric. Food Chem. 48 (2000) 4233.
[146] N.E. Es-Safi, V. Cheynier, M. Moutounet, J. Agric. Food Chem. 48 (2000) 5946.
[147] N.E. Es-Safi, H. Fulcrand, V. Cheynier, M. Moutounet, J. Agric. Food Chem. 47 (1999) 2096.
[148] N.E. Es-Safi, V. Cheynier, M. Moutounet, J. Agric. Food Chem. 50 (2002) 5571.
[149] H. Fulcrand, T. Doco, N.E. Es-Safi, V. Cheynier, M. Moutounet, J. Chromatogr. A 752 (1996) 85.
[150] N.E. Es-Safi, V. Cheynier, M. Moutounet, J. Agric. Food Chem. 50 (2002) 5586.
[151] C. Benabdeljalil, V. Cheynier, H. Fulcrand, A. Hakiki, M. Mosaddak, M. Moutounet, Sci. Aliments 20 (2000) 203.
[152] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutounet, Phytochemistry 47 (1998) 1401.
[153] N.E. Es-Safi, E. Meudec, C. Bouchut, H. Fulcrand, P.H. Ducrot, G. Herbette, V. Cheynier, J. Agric. Food Chem. 56 (2008) 4584.
[154] M. Schwarz, G. Jerz, P. Winterhalter, Vitis 42 (2003) 105.
[155] M. Schwarz, T.C. Wabnitz, P. Winterhalter, J. Agric. Food Chem. 51 (2003) 3682.
[156] M. Schwarz, G. Hofmann, P. Winterhalter, J. Agric. Food Chem. 52 (2004) 498.
[157] M. Gargouri, B. Gallois, J. Chaudiere, Arch. Biochem. Biophys. 491 (2009) 61.
[158] P. Alberts, M.A. Stander, S.O. Paul, A. de Villiers, J. Agric. Food Chem. 57 (2009) 9347.
[159] W.J. du Toit, K. Lisjak, M. Stander, D. Prevoo, J. Agric. Food Chem. 55 (2007) 2765.
[160] D. Fracassetti, N. Lawrence, A.G.J. Tredoux, A. Tirelli, H.H. Nieuwoudt, W.J. du Toit, Food Chem. 128 (2011) 1136.

[161] D. Rossouw, A.H. van den Dool, D. Jacobson, F.F. Bauer, Appl. Env. Microbiol. 76 (2010) 3911.
[162] H. Barnard, A.N. Dooley, G. Areshian, B. Gasparyan, K.F. Faull, J. Arch. Sci. 38 (2011) 977.
[163] T. Kandl, S. Kupina, Am. J. Enol. Vitic. 50 (1999) 155.
[164] G. Vanhoenacker, A. de Villiers, K. Lazou, D. De Keukeleire, P. Sandra, Chromatographia 54 (2001) 309.
[165] A. de Villiers, F. Alberts, F. Lynen, A. Crouch, P. Sandra, Chromatographia 58 (2003) 393.
[166] V.C. Galpin, A comparison of the legislation about wine-making additives and processes, Cape wine master diploma assignment, South Africa (2006).
[167] Compendium of international methods of wine and must analysis, Office International de la Vigne et du Vin (OIV), Paris, France (2002).
[168] M.D.L. de Castro, J. Gonzalez-Rodriguez, P. Perez-Juan, Food Rev. Int. 21 (2005) 231.
[169] B.W. Zoecklein, K.C. Fugelsang, B.H. Gump, F.S. Nury, Production wine analysis, Chapman & Hall, New York, USA (1990).
[170] P.J. Fletcher, J.F. van Staden, Anal. Chim. Acta 499 (2003) 123.
[171] B. Ayestarán, Z. Guadalupe, D. León, Anal. Chim. Acta 513 (2004) 29.
[172] P. Alberts, M.A. Stander, A. de Villiers, S. Afr. J. Enol. Vitic. 32 (2011) 51.
[173] P. Sandra, B. Tienpont, F. David, J. Chromatogr. A 1000 (2003) 299.
[174] A.E.M.M.R. Afify, M.A. Mohamed, H.A. El-Gammal, E.R. Attallah, Int. J. Food Agric. Env. 8 (2011) 602.
[175] P. Alberts, M.A. Stander, A. de Villiers, Food Add. Cont. A 28 (2011) 826.
[176] M. Waldner, O.P.H. Augustyn, Wineland, South Africa (2005) 98
[177] H.Y. Aboul-Enein, O.B. Kutluk, G. Altiokka, M. Tuncel, Biomed. Chromatogr. 16 (2002) 470.
[178] G.S. Shephard, A. Fabiani, S. Stockenstrom, N. Mshicileli, V. Sewram, J. Agric. Food Chem. 51 (2003) 1102.
[179] M.A. Stander, P.S. Steyn, S. Afr. J. Enol. Vitic. 23 (2002) 9.
[180] S. Lasram, N. Belli, S. Chebil, Z. Nahla, M. Ahmed, V. Sanchis, A. Ghorbel, Int. J. Food Microbiol. 114 (2007) 376.
[181] A. Selouane, D. Bouya, A. Lebrihi, C. Decock, A. Bouseta, J. Microbiol. 47 (2009) 411.
[182] A. Zinedine, J. Manes, Food Control 20 (2009) 334.
[183] N.E. Es-Safi, E.M. Essassi, M. Massoui, J. Banoub, Mass spectrometry as a powerful analytical technique for the structural characterization of synthesized and natural products, NATO Science for peace and security series A: Chemistry and Biology (2011), p 319 - 360.

Chapter 5

Development of a fast, sensitive and robust LC-MS/MS method for the analysis of natamycin in wine *

* Published as P. Alberts, M.A. Stander, A. de Villiers, S.A. J. Enol. Vitic. 32 (2011) 51-59.

5.1. Introduction

Natamycin, also known as pimaricin, is a macrolide polyene antifungal agent that specifically inhibits the growth of moulds and yeasts (Figure 5.1) [1,2]. Natamycin occurs naturally and is produced during fermentation by the bacterium *Streptomyces natalensis*, commonly found in soil. Due to the amphiphilic nature of the molecule it has low solubility in water, but it is effective at low concentrations, exhibits a wide spectrum of activity and has a neutral flavour impact – characteristics that render it ideally suited as a preservative [1,2]. Natamycin and its metabolites lack acute toxicity, but its use as a preservative should be considered in the light of its potential for antimicrobial resistance [2,3].

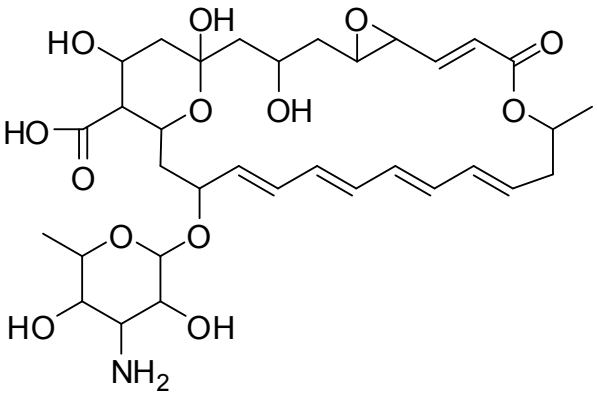


Figure 5.1. Chemical structure of natamycin ($C_{33}H_{47}NO_{13}$).

Natamycin is permitted as an antimicrobial preservative in more than 70 countries, mainly for processed meat and cheese products. In South Africa it is allowed as a preservative in a wide range of products, including wine [2]. South African legislation, however, explicitly forbids its use in liquor products intended for export to markets where it is prohibited [4]. As natamycin is forbidden in wine in the European Union (EU), wine containing this compound may not be exported to that market. In order to ensure accessibility to the important EU market (~R18 billion annually; Department of Agriculture, Forestry and Fisheries) for South African exporters, it is imperative that methodologies be developed to demonstrate compliance of export liquor products with relevant EU directives.

Natamycin is routinely analysed in a wide variety of foodstuffs, where its use is allowed, by spectrophotometric methods and/or a simple extraction step followed by reversed phase high performance liquid chromatography (RP-HPLC) [5,6]. However, neither of these methods provides the required sensitivity or selectivity for the analysis of this compound in the complex wine matrix at the regulatory levels. European Union regulations stipulate that natamycin should be absent from wine, therefore regulatory levels are dictated by the capabilities of

analytical methods. Currently a level of < 0.005 mg/L is enforced in Germany (personal communication, German authorities).

The objective of this study was therefore, in the first instance, the development of rapid, sensitive and robust methodologies for the determination of natamycin in wine. In view of the high-throughput requirement, methodologies based on rapid sample preparation and fast analysis using ultra high pressure liquid chromatography (UHPLC) technology were developed. Specifically, the known benefits of small-particle packed columns and elevated pressure operation for fast liquid chromatographic (LC) separations are combined with the high selectivity inherent to tandem mass spectrometric (MS/MS) detection to ensure fast and accurate determination of the target analyte [7]. As part of this research, the degradation kinetics of natamycin in different matrices was investigated, in view of the relevance of the known instability of this compound [8,9] to its accurate analytical determination.

5.2. Materials and methods

5.2.1. Chemicals and standards

High purity chemicals and solvents were used throughout, except for natamycin which was a commercial grade mixture of 50% natamycin in glucose. Methanol was from Merck (Darmstadt, Germany); acetonitrile, ammonium formate, ethanol, erioglaucine, sodium hydroxide and tartaric acid from Sigma-Aldrich (Mulbarton, South Africa); and formic acid from Saarchem (Wadeville, South Africa). C18 solid phase extraction (SPE) cartridges (500 mg/3 mL) were from Agilent Technologies (Chemetrix, South Africa); and aminopropyl SPE cartridges (500 mg/3 mL) from Waters Corporation (Microsep, South Africa).

A natamycin stock standard was standardised spectrophotometrically utilising published molar absorptivity data [1] and the purity of the reference material was calculated from this information. Working standards were prepared by serial dilution of the stock standard with a solution of 40% methanol in water containing 0.5% formic acid. Wine samples for the recovery and degradation studies were fortified volumetrically by adding appropriate amounts of reference standards. Recovery of natamycin was determined by quantitative analysis of samples fortified at three levels with known amounts prior to sample preparation (n = 18). In the degradation study the amount of natamycin remaining was quantified relative to an erioglaucine standard, which remained completely stable for the duration of the study.

5.2.2. Sample preparation

The optimised C18 SPE sample clean-up procedure consisted of pre-conditioning the cartridge with 3 mL each of methanol and water, consecutively. An aliquot of 2 mL wine was loaded, followed by matrix removal with 3 x 1 mL of 40% methanol and 0.5% formic acid in water. The analyte was eluted (3 x 0.7 mL) with a solution of 60% acetonitrile and 0.5% formic acid in water, and the effluent collected in a 5 mL graduated tube. Typical volumes recovered were approximately 1.9 mL, which were then reconstituted to 2 mL with water.

The aminopropyl-based SPE procedure consisted of mixing together, in a small test tube, 0.8 mL wine and 0.8 mL of a solution of 50% methanol and 1% formic acid in water. The diluted sample was then passed through the SPE cartridge without any pre-conditioning. The effluent (~1 mL) was collected directly into a 1.8 mL autosampler vial for analysis.

5.2.3. Liquid chromatographic methods and instrumentation

An Agilent 1100 LC system (Agilent Technologies) fitted with quaternary pump, autosampler, column oven and ultraviolet-visible (UV/Vis) diode array detector (DAD) was used for the LC-UV analysis. Separations were performed in reversed phase mode employing a Phenomenex Luna phenyl-hexyl column (150 x 4.6 mm, 3 µm particle size) at 25°C. The mobile phase was a 2 mM ammonium formate in acetonitrile (solvent A) / 2 mM ammonium formate in water (solvent B) gradient. The gradient started at 10% solvent A, increasing linearly to 70% over 7.4 minutes, followed by re-equilibration for 2.6 minutes (total run-time 10 minutes). The flow rate was 1.0 mL/minute and variable injection volumes up to 100 µL were used for purified extracts. Spectrophotometric detection was performed at 305 nm and spectra between 210 and 400 nm were acquired.

For liquid chromatography – mass spectrometry (LC-MS/MS), a Waters Acquity UPLC system comprising a binary pump, vacuum degasser, autosampler, column oven and Micromass Xevo tandem quadrupole MS detector was used. Ionisation was performed using electrospray ionisation (ESI) in positive mode. Samples were separated on a Waters Acquity BEH phenyl-hexyl column (100 x 2.1 mm, 1.7 µm) at 50°C. Variable injection volumes in the partial loop mode were used with the maximum injection volume limited to 10 µL. An acetonitrile/0.01% acetic acid in water gradient was used at a flow-rate of 0.3 mL/minute, starting at 5% acetonitrile isocratic for 18 seconds, increased linearly to 65% acetonitrile over 3 minutes. A 24 second column clean-up step followed consisting of 95% acetonitrile at a flow-rate of 0.5 mL/minute. Re-equilibration was for 1.5 minutes at 0.3 mL/minute (total run-time 5 minutes). Natamycin eluted at 2.80 minutes under the described conditions. The column effluent was

therefore directed to the detector between 2.2 and 3.2 minutes only; the rest of the chromatographic run was vented to waste. The following multiple reaction monitoring (MRM) transitions were acquired, with the corresponding optimal collision energies given in parenthesis: *m/z* = 666.1 → 648.0 (10 eV), 503.0 (10 eV), 485.0 (14 eV) and 467.0 (12 eV). The ion *m/z* = 503.0 was the most intense transition and was therefore used as the quantifier, while *m/z* = 485.0 served as confirmatory qualifier. The source parameters were as follows: capillary voltage 3.7 kV, cone voltage 16 V and extractor voltage 3 V. The source and desolvation temperatures were 120ºC and 350ºC, respectively. The desolvation and cone gas flows were 600 and 60 L/hour, respectively.

5.3. Results and discussion

5.3.1. Sample preparation

Solid phase extraction is a versatile and efficient sample preparation technique offering a multitude of separations based on polar, hydrophobic and/or ion-exchange interactions [10]. Two SPE sample preparation strategies were developed and evaluated for suitability in this application. Sample pre-concentration was not pursued; instead, the emphasis was on removal of potential interferences while maintaining the matrix in a weak solvent relative to the intended separation in order to utilise the large loading capacity inherent to LC to achieve low detection levels.

Natamycin is hydrophobic and is strongly retained in reversed phase mode on C18 SPE cartridges. On this phase, acetonitrile has a higher eluting strength for natamycin compared to methanol. Selectivity could thus be optimised by effecting matrix removal with a relatively strong methanol-based solution, while the analyte is eluted with a relatively weak acetonitrile-based solution. Recovery of natamycin from a wine matrix with the optimised C18-based procedure was very good (~90%). However, wine phenolics were co-extracted under these conditions; red wine extracts retained a deep red colour.

In contrast, aminopropyl SPE phases had very little affinity for natamycin in a wine matrix. The ability of this phase to retain wine polyphenols was confirmed by experiments with red wine, where the retention of phenolics was evident from the retained pigments and their removal from treated samples. This offers the possibility of using the aminopropyl phase to remove the interfering wine matrix by direct elution of natamycin. The recovery of natamycin from a wine matrix was consistently >80%. Most visible pigments were removed from a red wine treated in this manner. This approach provides the critical benefit of simpler and faster

sample preparation, which is indispensable for fast routine analysis of large numbers of samples. Furthermore, the obtained sample is dissolved in a weak solvent compared to the starting conditions of RP-LC separations, thus facilitating large injection volumes without sacrificing chromatographic performance.

A comparison of the degree of sample matrix elimination achieved using each of these SPE methods is presented in Figure 5.2, where red wine extracts analysed with LC-UV are compared. Ultraviolet detection at 305 nm clearly illustrates that the aminopropyl-based SPE procedure provides a much cleaner extract. This is critical for LC-ESI-MS/MS analysis since ion suppression effects caused by co-eluting wine matrix elements (following C18 clean-up), are effectively eliminated by the aminopropyl-based SPE procedure. Importantly, the elimination of ion suppression during ionisation facilitated the quantitation of natamycin in wines with external standards, obviating the necessity for standard addition or isotope labelled internal standards for accurate quantitation. This procedure was therefore validated for LC-UV and LC-MS/MS determination of natamycin in wine.

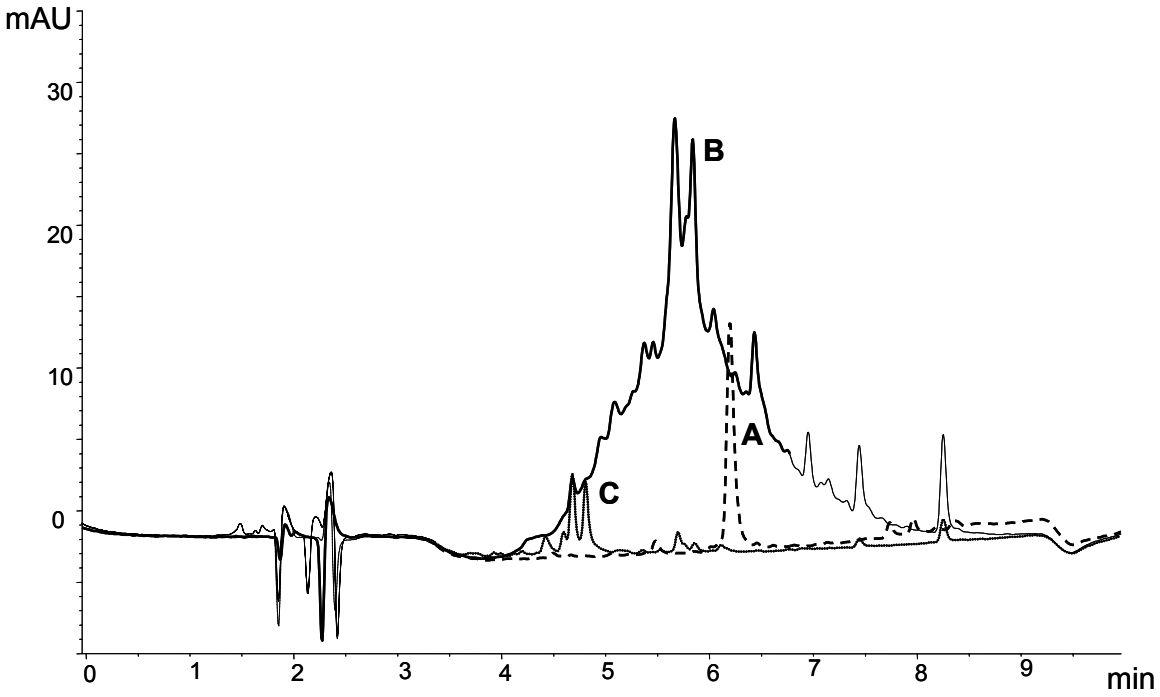


Figure 5.2. Overlay of LC-UV chromatograms of (A) 6.2 mg/L natamycin standard, (B) red wine sample after clean-up with the C18 SPE sample preparation procedure, and (C) the same wine following aminopropyl SPE clean-up. Injection volumes were 5 µL throughout.

5.3.2. HPLC-UV screening method for natamycin in wine

Reversed phase separation using a phenyl-hexyl column was used for LC-UV and LC-MS/MS determination of natamycin, because better retention was achieved compared to C18 phases due to aromatic selectivity offered by the phenyl-hexyl phase. Acetonitrile was selected as mobile phase organic modifier since higher separation efficiencies were obtained compared to methanol-based mobile phases. The use of 2 mM ammonium formate (pH 4.28) as acidic modifier in the mobile phase improved the chromatographic efficiency.

Natamycin contains strong chromophores and shows five maxima in its UV absorption spectrum (220, 280, 290, 303 and 318 nm). The most intense absorption is at 303 nm, displaying a relatively large molar absorptivity of 83220 $L.mol^{-1}.cm^{-1}$ [1]. These spectral features suggest that LC-UV may be applied successfully for the determination of low concentrations of natamycin. However, wine polyphenols such as flavonols, coumaric acids and anthocyanins also absorb in this region [11] and are expected to interfere with the LC-UV analysis of natamycin in wine. A sample preparation strategy capable of selectively removing wine polyphenols prior to analysis is therefore indispensable for trace-level analysis.

The optimised aminopropyl SPE sample clean-up procedure was therefore used in combination with LC-UV screening analyses. This procedure was validated. The LC-UV response was linear for injections of 0.1 to 520 ng natamycin on column (r = 0.9999). The limit of detection (LOD) and limit of quantitation (LOQ) of the method were 0.1 mg/L and 0.3 mg/L, respectively, for injection volumes of 100 µL of purified extracts. Spectral correlation with calibration standards was observed at sample concentrations >0.3 mg/L. The average recovery of natamycin from wine samples fortified at 10.4 mg/L, 1.04 mg/L and 0.208 mg/L was 89.3% (RSD 9.7%, *n* = 18).

Liquid chromatography with UV detection therefore presents a simple, inexpensive and robust methodology for the determination of natamycin in wine in the sub-parts per million range, while the wide availability of these systems makes this an ideal method for the screening of large numbers of samples. This method may be used to quantify natamycin in wines fortified with this compound for preservative purposes (for example wines intended for the local market). However, LC-UV does not possess the analytical sensitivity to meet the criteria for demonstrating conformity to EU regulations for exported wine. The method can not detect low concentrations of natamycin such as might result, for example, from secondary contamination.

5.3.3. UHPLC-MS/MS method for the trace-level quantitative determination of natamycin in wine

As the goal of this work was to develop a method suitable for the routine analysis of a large number of samples, an UHPLC column packed with 1.7 µm particles was used in order to exploit the well-known benefits of these particles for very fast analyses [7]. A phenyl-hexyl phase was selected for reasons outlined previously. Use of a 2.1 mm internal diameter column at an optimal flow rate of 0.3 mL/minute furthermore allows direct connection to ESI-MS without post-column splitting. For LC-MS/MS analyses, positive mode ESI was used, as atmospheric pressure chemical ionisation (APCI) of this large molecule had proved unsuccessful. Source conditions for MS detection were optimised by infusion of a 5 mg/L standard while adjusting experimental parameters to produce the best sensitivity. It was further established that ESI efficiency decreases with increasing levels of acid in the mobile phase. The acid content of the mobile phase was therefore minimised in favour of enhanced sensitivity at the cost of an acceptable reduction in the chromatographic performance. In order to optimise this relationship between the chromatographic efficiency and ionisation efficiency, acetic acid was introduced *via* the aqueous component of the mobile phase so that a relatively high amount is present initially (which minimise band broadening during the initial part of the separation) whereas the analyte is eluted in a fraction of the mobile phase that contains less acid to facilitate ionisation. An example of the optimised SPE-UHPLC-ESI-MS/MS analysis of a red wine sample spiked with 0.001 mg/L natamycin is presented in Figure 5.3.

The overall LC-MS/MS procedure is based on polar SPE sample clean-up, reversed phase chromatographic separation and MS/MS detection in MRM mode, and is therefore characterised by very good selectivity. Moreover, the relatively large molar mass (*m/z* of M + 1 = 666.1) and high-mass daughter ions (*m/z* 503.0 and 485.0) further increases the selectivity of the complete analytical procedure, rendering the method free of interferences (Figure 5.3).

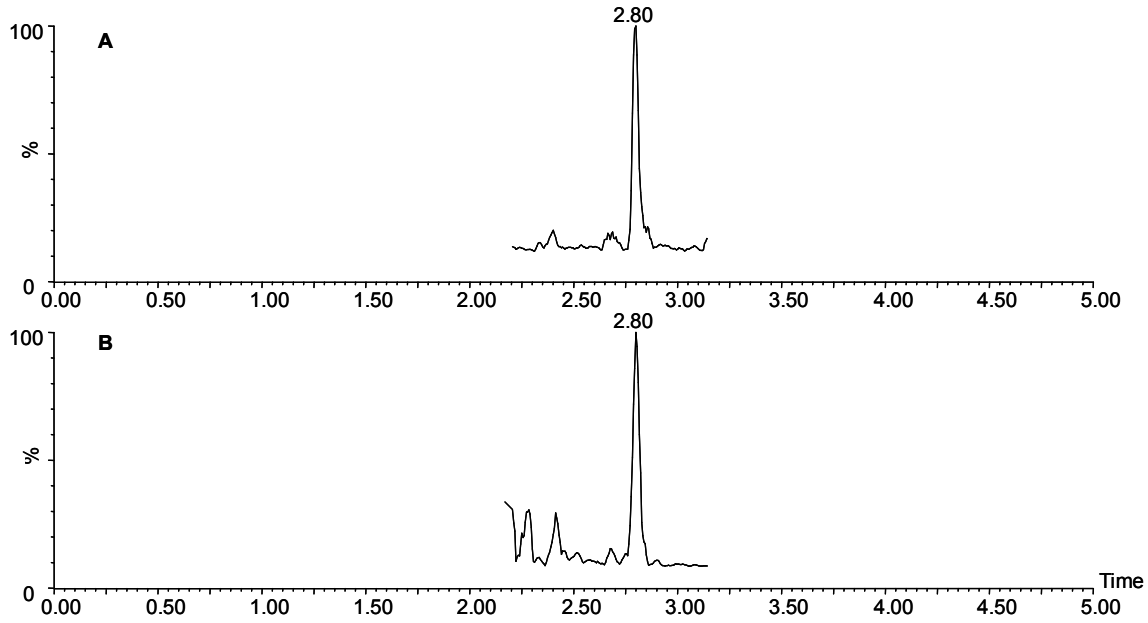


Figure 5.3. Multiple reaction monitoring chromatograms of two ion transitions (*m/z* = 666.1 → 485.0 (A) and *m/z* = 666.1 → 503.0 (B)) obtained for the SPE-UHPLC-MS/MS determination of natamycin in a spiked red wine sample containing 0.001 mg/L (injection volume 10 µL).

Validation of the optimised method showed a linear response over the range 0.002-20 ng (r = 0.9999). The LOD and LOQ (calculated as the smallest amount of solute that produced a signal equivalent to three and ten times the average noise, respectively, for unsmoothed chromatograms) were determined as 1.5 pg and 5.0 pg on column, respectively. These values translate to LOD and LOQ values of 0.0003 mg/L and 0.001 mg/L, respectively, considering that the samples were diluted 1:1 during sample preparation and that injection volumes of 10 µL are routinely used. This level of sensitivity is better than required for regulatory purposes (currently enforced at < 0.005 mg/L by Germany). Identification is based on retention time and two ion transitions (*m/z* = 666.1 to 503.0 (primary) and 666.1 to 485.0 (secondary)). The ratio of abundance of these transitions (1.6) was used to conclusively identify the analyte.

The accuracy and reproducibility of the method was assessed by determination of the recovery of natamycin from red wine samples fortified to 0.117 mg/L, 0.023 and 0.009 mg/L, respectively. The average recovery of the analytical method was 82.5% (RSD 6.6%, *n* = 18). Method repeatability, evaluated by eight repeated injections of a 0.04 mg/L standard solution, was 4.1% RSD for peak area. The intra-assay precision was assessed from the repeatability data obtained in the recovery study. Uncertainty of measurements associated with the LC-MS/MS procedure was estimated by the 95% confidence interval about the

measurements performed in the recovery study and was calculated as ± 2.6%. As the LOQ of the procedure is 0.001 mg/L, and the recoveries of the order 82.5 ± 2.6%, results are reported in units of mg/L by recording three significant figures.

5.3.4. Degradation kinetics of natamycin in the wine matrix

Natamycin is unstable in solution at both low and high pH, and the stability is further affected by temperature, light exposure and oxidation [8]. However, no data on the stability of this compound in wine have been reported. Under acidic conditions (such as encountered in wine), natamycin is degraded rapidly *via* hydrolysis of the glycosidic bond to yield mycosamine and various other products [8,9,12]. The fact that the compound is labile under conditions encountered in the wine matrix clearly has implications for the validity of quantitative results, since its concentration is expected to decrease with time. For this reason the degradation of natamycin in red, white and synthetic wine matrices was studied. The synthetic wine matrix consisted of a solution of 12% ethanol and 2.5 g/L tartaric acid in water (pH adjusted to 3.5 with sodium hydroxide). Four ~4L batches (two red, one white and one synthetic) were fortified with ~50 mg/L natamycin. These were each divided into four sub-sets (in 750 mL bottles), kept at 20°C (light and dark, respectively), 30°C and 40°C. Natamycin was quantified using the LC-UV method described above to study degradation kinetics. At the fortification levels used here, small injection volumes of neat wine could be employed, thereby obviating sample pre-treatment. Results for one batch of red wine are shown in Figure 5.4. An exponential relationship was found to apply in all cases, corresponding to pseudo first-order degradation. Confirmation of the first-order kinetics was obtained graphically from the linearity of plots of *ln* concentration against time (correlation coefficients, r, were between 0.999 and 0.975). The rate constant, *k*, was calculated from the first-order rate equation

$$c_t = c_0 e^{-kt}$$

where c_t represents the concentration of natamycin remaining at any time t, c_0 the initial concentration and k the rate constant for the degradation reaction in days^{-1} [13].

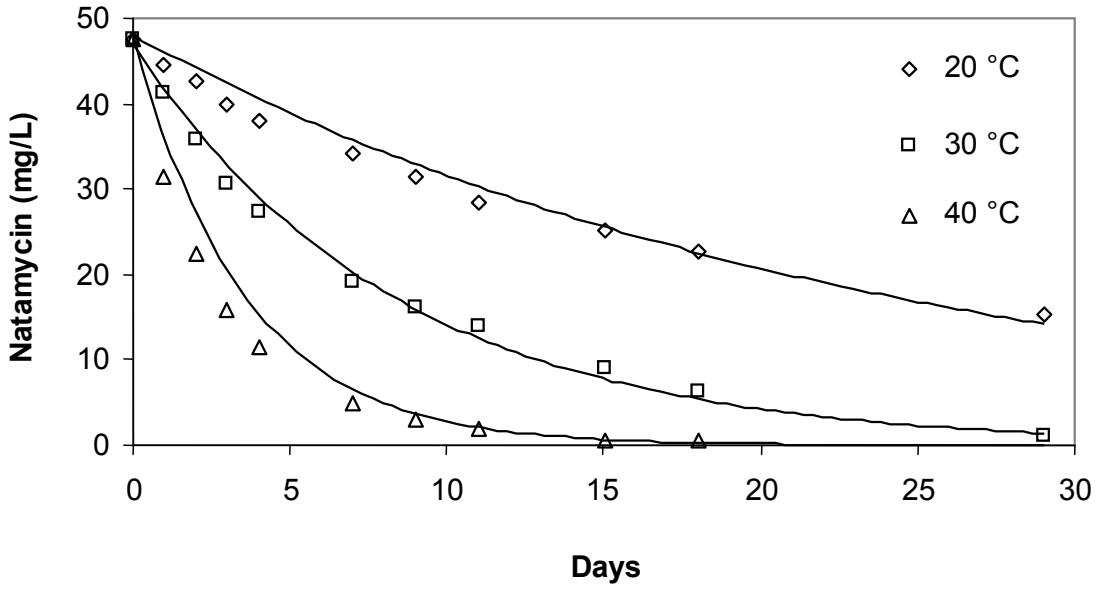


Figure 5.4. Degradation of natamycin in a red wine matrix at different temperatures.

Half-lives for each dataset were determined from the following equation [13]:

$$t_{1/2} = \frac{\ln 2}{k}$$

Results for degradation kinetics for Natamycin in wine are summarised in Table 5.1. The activation energy (E_a) for the degradation reaction involving natamycin in wine was calculated using the Arrhenius equation [13]:

$$\ln k = \ln A - \frac{E_a}{RT}$$

where *A* is the pre-exponential factor and *R* is the gas constant (= 8.3143 $J.K^{-1}.\ mol^{-1}$). Plots of *ln k* vs *1/T* provided linear relationships (r = 0.994-1.000, Figure 5.5), from which the activation energies reported in Table 5.2 were calculated.

Table 5.1. Kinetic parameters for the degradation of natamycin in wine (*n* = 4).

Batch	Temp. (°C)	Regression equation	Rate constant (k, days^{-1})	Half-life ($t_{1/2}$, days)
Red wine[a]	20	$c = 45.4e^{-0.03868t}$	0.03868	17.9
White wine[b]	20	$c = 38.9e^{-0.05899t}$	0.05899	11.8
Red wine[c]	20	$c = 41.4e^{-0.03378t}$	0.03378	20.5
Synthetic matrix[d]	20	$c = 52.8e^{-0.01443t}$	0.01443	48.0
Red wine[a]	30	$c = 45.1e^{-0.1202t}$	0.1202	5.8
White wine[b]	30	$c = 35.9e^{-0.1592t}$	0.1592	4.4
Red wine[c]	30	$c = 45.5e^{-0.1216t}$	0.1216	5.7
Synthetic matrix[d]	30	$c = 52.4e^{-0.03605t}$	0.03605	19.2
Red wine[a]	40	$c = 40.0e^{-0.2861t}$	0.2861	2.4
White wine[b]	40	$c = 33.8e^{-0.4368t}$	0.4368	1.6
Red wine[c]	40	$c = 37.1e^{-0.2735t}$	0.2735	2.5
Synthetic matrix[d]	40	$c = 53.7e^{-0.1151t}$	0.1151	6.0

[a] pH = 3.55

[b] pH = 3.49

[c] pH = 3.67

[d] pH = 3.50

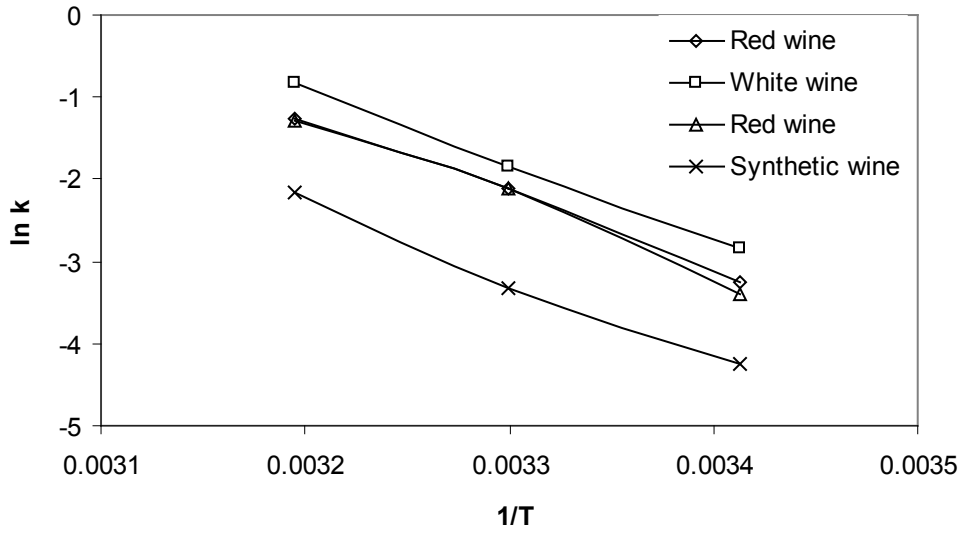


Figure 5.5. Arrhenius plots for the degradation of natamycin in wine matrices.

Table 5.2. Parameters for the determination of the activation energy (E_a) for the degradation of Natamycin in wine.

Batch	Temp (°C)	*ln k*	*1/T* (K^{-1})	Activation energy, E_a ($kJ.mol^{-1}$)
Red	20	-3.252	0.00341	76
wine	30	-2.119	0.00330	
	40	-1.251	0.00319	
White	20	-2.830	0.00341	76
wine	30	-1.838	0.00330	
	40	-0.8283	0.00319	
Red	20	-3.388	0.00341	79
wine	30	-2.107	0.00330	
	40	-1.296	0.00319	
Synthetic	20	-4.238	0.00341	78
matrix	30	-3.323	0.00330	
	40	-2.162	0.00319	

These results clearly confirm that natamycin is unstable in the wine matrix under normal storage conditions. The relatively low activation energies (~80 $kJ.mol^{-1}$) indicate that the degradation reaction proceeds readily, even at typical wine storage temperatures. The half-life for the degradation of natamycin in wine is approximately 20 days at 20°C, 6 days at 30°C and 2 days at 40°C (Table 5.1). The degradation reaction proceeded faster in white wine compared to red wine, which may be ascribed to the higher acidity of the white wine (pH of 3.49 compared to pH 3.55 and 3.67 of the two red wines used here). Similar degradation rates were observed at 20°C in these matrices for batches stored in darkness and artificial light (results not shown). Since the samples were kept in wine bottles, radiation may have been reduced sufficiently by absorption of the glass so that artificial light had no effect on the rate of degradation. The data reported here may then be considered representative of the stability of natamycin under normal wine storage conditions.

Several degradation products were noted in LC-UV chromatograms of natamycin in a synthetic wine matrix stored at elevated temperatures (Figure 5.6). These are all more polar than natamycin as they eluted earlier in the reversed phase separation. Ultraviolet spectra of

these degradation products were identical to that of intact natamycin, indicating that the degradation products retain the tetraene structure of the original molecule. This phenomenon was also noted in an earlier study [8]. No accumulation of any specific break-down product was noted concurrently with degradation of natamycin in the synthetic matrix. Analysis of the same sample using LC-MS (results not shown) allowed the tentative identification of a variety of breakdown products, including mycosamine (the amino sugar degradation product of natamycin) as well as several isomers of auto-oxidation and hydrolysed (presumably at the epoxy group) products [8]. Further work is required for the detailed elucidation of the relevant degradation reactions of natamycin in wine.

Factors responsible for natamycin degradation in calibration standard solutions were also investigated by evaluating stability in various matrices at 20°C. It was found that the addition of formic acid in the range of 0 (pH 3.5), 1 (pH 2.3), 5 (pH 1.9) and 10% (pH 1.6) to a series of standards prepared in 10% methanol caused a drastic acceleration in the degradation of the compound. Acidification (minimum of 0.5% formic acid with 40% methanol in water) of standards is, however, indispensable to ensure solubility in solvents equivalent to the starting conditions of reversed phase separation. Natamycin is soluble at ~50 µg/mL in water [12]. Moreover, it was found that natamycin stability increases with methanol content in a series of standards prepared in 5, 10, 20 and 40% methanol (constant acidity). These findings are consistent with a predominantly hydrolytic degradation mechanism as degradation is generally faster at lower pH and higher aqueous content, respectively.

For these reasons, all standard solutions were prepared in 40% methanol and 0.5% formic acid in water. Standard solutions were stored at 4°C while new standards were prepared monthly.

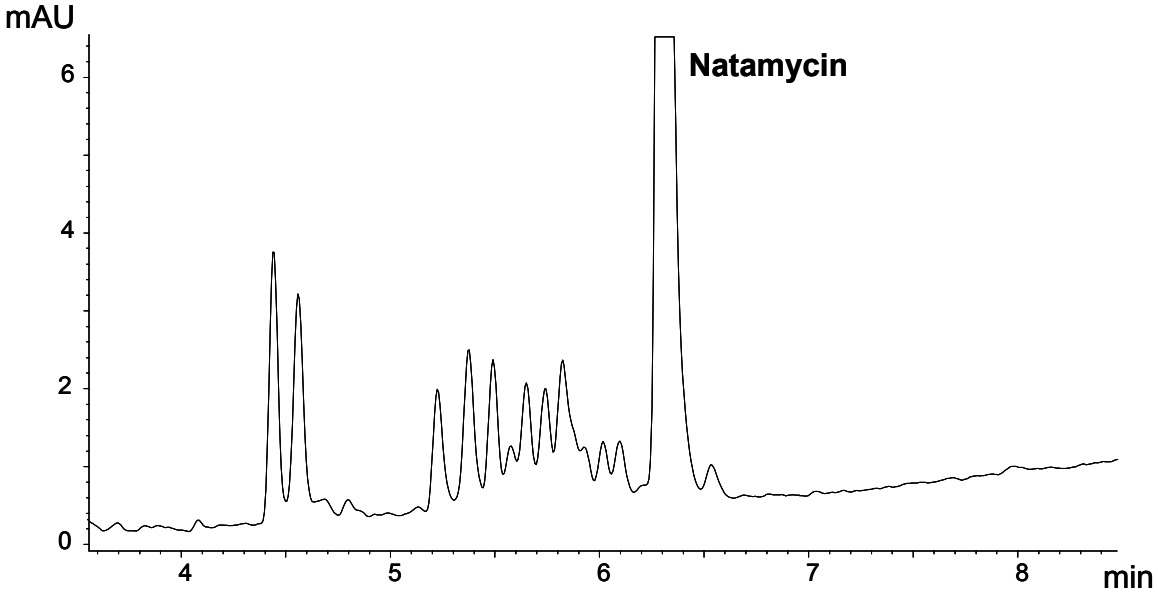


Figure 5.6. LC-UV chromatogram of analysis of natamycin in a synthetic wine matrix stored for 18 days at 30°C illustrating the degradation products.

5.4. Conclusions

Natamycin is not a natural constituent of wine and should therefore be absent from the product where it is prohibited. The presence of natamycin in wine may be due to direct addition for preservation purposes (for example in countries where this practice is allowed), or as a result of secondary contamination. Sources of secondary contamination include the addition of sweet must to the product, as well as contamination during processing, bottling and ageing of the product (for example, natamycin has been detected in some imported European compound corks). Local wine producers should be made aware of this risk, and take all possible measures to avoid possible sources of secondary contamination in wines intended for export. Although the resulting levels of natamycin would be below the effective concentration of the substance, it would nevertheless result in rejection of products from the European market.

In terms of the analytical quantitation of Natamycin in wine, the latter condition clearly places a high demand on method sensitivity. The WHO regards exposure to antimicrobials at sub-effective levels as a contributing factor in the spread of antimicrobial resistance [3], while German authorities implement acceptance levels of < 0.005 mg/L natamycin in wine. Suitable analytical methods are therefore required to demonstrate compliance and facilitate international wine trade. Another relevant requirement is for methods to be sufficiently fast and robust for the high throughput routine analysis of large numbers of samples.

In this study, methods based on HPLC analysis in combination with SPE sample clean-up were developed for the determination of natamycin in wine. Liquid chromatography with UV detection is an accurate, robust and inexpensive analytical methodology, although not sufficiently sensitive to demonstrate compliance with EU standards. The LC-MS/MS procedure developed in this study is very sensitive and also provides qualitative information for positive compound identification. The use of dedicated ultra-high pressure columns and instrumentation provides very rapid analyses. In combination with an optimised SPE method, the overall procedure is fast and sufficiently specific to be rendered free from interference from the wine matrix. The developed methodology therefore produces performance specifications well within the requirements and is ideally suited for routine application to demonstrate compliance of wines with EU directives concerning natamycin.

Furthermore, degradation studies revealed that natamycin decomposes readily under normal wine storage conditions. The half-life in wine is of the order of 20 days at 20°C, and the activation energy for the decomposition is ~80 $kJ.mol^{-1}$. The fact that the substance is labile in the wine matrix complicates accurate quantitation, since time as well as storage conditions (mainly temperature) affect its concentration. Aqueous calibration standards are also unstable and precautions such as cold-storage and regular preparation of fresh standards are required for accurate quantitation.

References

[1] S. Budavari, The Merck Index: An encyclopaedia of chemicals, drugs and biologicals, Merck & Co. Inc., New Jersey, U.S.A. (1989).
[2] Food Standards Australia New Zealand (FSANZ), Application A542, Natamycin – extension of use as a food additive (2004).
[3] World Health Organization, Fact sheet No. 194 (2002).
[4] Liquor Products Act, Government Gazette of the Republic of South Africa, Act 60 (1989).
[5] L.F. Capitan-Vallvey, R. Checa-Moreno, N. Navas, J. AOAC Int. 83 (2000) 802.
[6] W. de Ruig, J.J. Van Oostrom, K. Leenheer, J. Assoc. Off. Anal. Chem. 70 (1987) 944.
[7] A. de Villiers, F. Lestremau, R. Szucs, S. Gélébart, F. David, P. Sandra, J. Chromatogr. A 1127 (2006) 60.
[8] H. Brik, J. Antibiotics (1976) 632.
[9] J.L. Koontz, J.E. Marcy, W.E. Barbeau, S.E. Duncan, J. Agric. Food Chem. 51 (2003) 7111.
[10] E.M. Thurman, M.S. Mills, Solid-phase extraction. Principles and practice. Chemical analysis: A series of monographs on Analytical Chemistry and its applications, John Wiley & Sons, Vol. 147 (1998).
[11] B. Abad-Garcia, L.A. Berrueta, S. Garmon-Lobato, B. Gallo, F. Vicente, J. Chromatogr. A 1216 (2009) 5398.
[12] J.C. Pedersen, Applied and Environmental Microbiology (1992) 1064.
[13] P.W. Atkins, Physical Chemistry, Oxford University Press, Oxford, U.K. (1998).

Chapter 6

Development of a novel solid phase extraction – liquid chromatography – mass spectrometry method for the analysis of ethyl carbamate in alcoholic beverages: Application to South African wine and spirits *

* Published as P. Alberts, M.A. Stander, A. de Villiers, J. Food Add. Contam. A 28 (2011) 826-839.

6.1. Introduction

Ethyl carbamate (EC) is a known genotoxic carcinogen which occurs naturally in fermented food and alcoholic beverages, including spirits, wine and beer [1-3]. In 2005, the Joint Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO) Expert Committee on Food Additives (JECFA) concluded that alcoholic beverages constitute a major source of EC ingestion in humans [4]. In 2007, the International Agency for Cancer Research (IARC) reassessed the carcinogenicity of alcoholic drinks and consequently upgraded EC from a group 2B substance ('possibly carcinogenic to humans') to a group 2A substance ('probably carcinogenic to humans') [5]. Due to its potential carcinogenic effects, several countries have implemented legislation that limits the EC content of alcoholic beverages [6,7]. These limits range from 15 µg/L in wine (recommended in USA) to 1 mg/L in fruit brandy (France and Switzerland) [7-9].

Since the presence and associated health risks of EC in alcoholic beverages became known, measures have been instigated globally to reduce the risk of contamination in these commodities [2-4,10-12]. The formation of EC in alcoholic beverages may be minimised *via* two general approaches: by restricting the formation of the main precursors and by limiting their tendency to react to yield EC [4,7]. Examples of such preventative actions include design changes in the bourbon production processes and implementation of 'Integrated Production' schemes in the global wine industry to ensure sustainable production [2,3,7].

In light of the above, it is imperative that rapid and reliable analytical methodologies are available for EC determination in alcoholic beverages to minimise the risk to consumers. Moreover, since maximum levels are regulated internationally, demonstration of compliance of export products may become a prerequisite for trade in future.

Current routine analytical methods used for EC determination employ liquid-liquid extraction (LLE) [13,14] and solid phase extraction (SPE) [15-18] for sample preparation, followed by gas chromatography – mass spectrometry (GC-MS) [13,15,16] or gas chromatography – nitrogen-phosphorus detection (GC-NPD) [17] analysis. Headspace solid phase micro-extraction (HS-SPME) coupled to GC-MS [19] or tandem quadrupole mass spectrometric detection (HS-SPME-GC-MS/MS) [20] as well as comprehensive two-dimensional GC coupled to time-of-flight mass spectrometry (HS-SPME-GCxGC-TOF) [6] have also been used for EC determination. Quantitative GC analyses generally involve internal standards such as *n*-propyl carbamate [15,17], cyclopentyl carbamate [16] and deuterated or isotope labelled EC [18,20]. The official method of analysis adopted by the *Office International de la Vigne et du Vin* (OIV) utilises propyl carbamate as internal standard, SPE on diatomaceous earth columns

and GC-MS analysis in selected ion monitoring (SIM) mode [21]. Liquid chromatographic (LC) procedures involving pre-column derivatisation with 9-xanthydrol, reversed phase (RP) separation and fluorescence detection [22] as well as LC-MS/MS [23] have also been reported for EC analysis.

Sample preparation is especially important for the analysis of EC in alcoholic beverages. Published SPE methods utilise ethanol removal via centrifugation under vacuum [18,24] or involve large capacity diatomaceous earth columns [15,17]. These methods are labour-intensive and time-consuming and in addition, the diatomaceous earth cartridges require large volumes of toxic dichloromethane for elution of the analyte. Moreover, because EC sublimes [1], internal standards are required to compensate for volatility losses of the analyte. liquid-liquid extractions inherently use relatively large volumes of solvent [13,14], require evaporative removal of the solvent and produce relatively low separation efficiencies. Matrix interferences in the determination of EC in wines at low concentrations utilising these sample preparation strategies have also been reported [14,17]. Methods that employ direct injection of samples [6,20,22] yield relatively high detection levels, which render them unsuited to study the occurrence of EC in diverse commodities (especially wine).

The principal objective of this study was therefore to develop a simple, rapid and robust procedure for trace-level determination of EC in wines and spirits, with none of the drawbacks associated with currently utilised methods. An SPE sample pre-treatment step was developed for this purpose, and utilised in combination with LC-MS/MS for the quantitative analysis of EC in diverse alcoholic beverages. The developed method was validated and applied to study the occurrence of EC in South African wines and spirits and the factors responsible for the formation of EC in these commodities.

6.2. Experimental

6.2.1. Materials

Acetone (Pestanal grade) was obtained from Fluka (Seelze, Germany), ethyl carbamate from Sigma-Aldrich (Mulbarton, South Africa), ethyl acetate (HPLC grade) from Riedel-de Haën (Seelze, Germany), isooctane (HiPerSolv for HPLC grade) from BDH (Poole, England), sodium chloride from Thomas Baker Chemicals (Mumbai, India) and ethanol from Merck (Darmstadt, Germany). Oasis HLB SPE cartridges (500 mg/6 mL) were obtained from Waters Corporation (Milford, U.S.A.). Samples were obtained from import and export applications (South African Department of Agriculture, Forestry and Fisheries).

Calibration standards were prepared in 75% ethyl acetate in isooctane. Standards used in the recovery study were diluted in a solution of 50% ethanol in water. Wine samples for the recovery study were fortified volumetrically by adding appropriate quantities of standards. For the study of the effect of alcohol content on EC formation, a batch of wine was sub-divided and fortified by adding various quantities of ethanol and water to produce a series of samples with a range of alcohol concentrations with a constant wine matrix. For the study of the effect of pH on EC formation, sample pH was adjusted by adding small quantities of formic acid and/or ammonium hydroxide solutions (Merck).

6.2.2. Sample preparation

An Oasis HLB SPE cartridge was conditioned by consecutively aspirating 5 mL methanol, water and a saturated sodium chloride solution through the column. Sodium chloride (~1 g) was dispensed onto the cartridge concurrently with aspiration of the saturated sodium chloride solution. Natural wine samples (alcohol content <15%) were diluted by adding 2.5 mL wine to 2.5 mL saturated sodium chloride solution (the final alcohol content of the diluted samples should not exceed ~7.5%). Fortified wine samples (alcohol content <20%) were diluted by adding together 2.0 mL wine and 3.0 mL saturated sodium chloride solution, while spirit samples (alcohol content <45%) were diluted by adding 1.0 mL sample to 4.0 mL saturated sodium chloride solution. Diluted samples (~5 mL) were loaded, followed by drying of the column under reduced pressure for 1 minute. The column was rinsed with isooctane (3 x 1 mL) and dried again for 1 minute. The analyte was eluted with 3 x 0.9 mL 80/20 ethyl acetate/isooctane and collected in a 5 mL graduated tube. Typical volumes recovered were ~2.3 mL, which were diluted to 2.5 mL with isooctane. The ethyl acetate/isooctane phase was mixed, centrifuged (30 seconds) and a 1 mL aliquot was removed for analysis.

6.2.3. Liquid chromatographic methods and instrumentation

Normal phase liquid chromatography tandem quadrupole mass spectrometric (NP-LC-MS/MS) analyses: A Waters Alliance 2695 LC system (Waters Corporation, Milford, U.S.A.) incorporating a quaternary pump, vacuum degasser, autosampler, column oven and Micromass Quattro Premier XE tandem quadrupole MS detector (Manchester, U.K.) equipped with an APCI probe (Micromass IonSabre APCI probe) was used. Sample extracts were separated in normal phase (NP) mode using a Phenomenex Luna aminopropyl column (250 x 4.6 mm, 5 µm particle size, Torrance, U.S.A.) thermostatted at 40ºC. Variable injection volumes were used, with the maximum injection volume limited to 100 µL. The mobile phase was an acetone/isooctane gradient: 15-40% acetone in 11 minutes, 40-99% acetone in 0.9

minutes. The column was re-equilibrated for 3 minutes (total run-time 15 minutes) and the flow-rate was 1.0 mL/minute throughout. Ethyl carbamate eluted at 10.7 minutes under these conditions. The column effluent was directed to the detector between 9.5 and 12.5 minutes only; the rest was vented to waste. Data acquisition was in multiple reaction monitoring (MRM) mode, acquiring data for a single secondary ion transition (*m/z* = 90.1 ® 62.3). The optimal collision energy was 8 eV and source parameters as follows: corona needle current 6 µA, cone voltage 16 V, extractor voltage 6 V and RF lens 0 V. The source and desolvation temperatures were 110ºC and 100ºC, respectively. The desolvation and cone gas flows were 150 and 100 L/hour, respectively.

Reversed phase liquid chromatography with ultraviolet detection (RP-LC-UV) analyses: The LC-UV analyses were preformed on an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) fitted with a quaternary pump and diode array detector. Reversed phase analyses were performed on a Phenomenex Luna C18 column (150 x 4.6 mm, 3 µm), thermostatted at 40ºC. The injection volume was 5 µL. An acetonitrile/water gradient starting at 10% acetonitrile and increasing to 90% in 25 minutes followed by re-equilibration for 4 minutes was used (total run-time 29 minutes). The flow rate was 1.0 mL/minute. UV detection was performed at 200 nm.

6.3. Results and discussion

6.3.1. Development of an SPE method for sample clean-up

The determination of EC in alcoholic beverages presents an analytical challenge due to low analyte concentrations (in the µg/L range) and the complexities of the matrix. In particular, sample clean-up and pre-concentration is complicated by the fact that the compound is highly polar and hydrophilic [1], and therefore difficult to separate from the aqueous alcoholic matrix. Methods described in literature for these analyses suffer from several drawbacks, including time-consuming sample pre-treatment, use of large volumes of toxic solvents and the requirement of internal standard quantification. Therefore, a novel SPE method was developed, mindful of two intrinsic advantages associated with LC-MS/MS: the large sample loading capacity of LC combined with the inherent sensitivity and selectivity of MS/MS (in MRM mode) were exploited to simplify the sample preparation strategy. Pre-concentration was therefore not pursued; instead, emphasis was on sample clean-up while maintaining the sample in a weak solvent relative to the intended separation in order to exploit the high loading capacity inherent to LC.

Initial experiments showed that alcoholic samples preclude conventional RP SPE strategies (C18, Oasis HLB and SDB), because EC is not retained efficiently, even after reduction of the ethanol content by dilution. Moreover, normal phase SPE was found to be inherently incompatible with sample matrices encountered in alcoholic beverages (essentially ethanol/water mixtures). Aminopropyl, silica and Florisil phases did not provide sufficient retention of EC in ethyl acetate extracts of wine samples. More apolar solvents such as isooctane and di-ethyl ether failed to extract EC efficiently from a wine matrix (dichloromethane was not evaluated on account of its toxicity).

The Oasis HLB SPE phase possesses hydrophilic-lipophilic properties under RP conditions. This property was exploited by inducing EC retention through an increase in ionic strength of the sample matrix *via* saturation with sodium chloride. Matrix removal was achieved by rinsing with isooctane, followed by elution of EC using a solution of ethyl acetate in isooctane. This strategy produced quantitative recovery of EC from all matrices and yielded clean extracts that are inherently compatible with normal phase LC. Moreover, the extract is a relatively weak NP-LC solvent, thereby facilitating the use of large injection volumes without compromising chromatographic performance.

The extent of sample matrix elimination achieved with this procedure is demonstrated by the analysis of a red wine and the SPE extract of the same wine by RP-LC-UV using non-specific detection at 200 nm. The overlaid chromatograms are shown in Figure 6.1; it is clear that the SPE procedure successfully eliminates the majority of wine interferents. Ethyl carbamate is not detected under these conditions due to the lack of a chromophore, but from experiments with this elution system on LC-MS, it is known to elute close to the void volume.

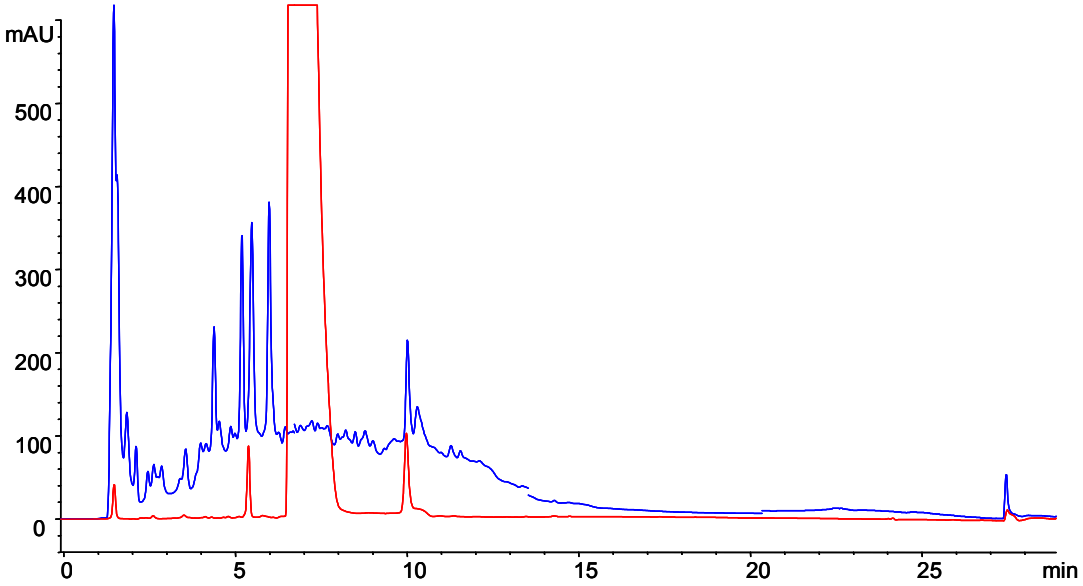


Figure 6.1. Overlay of HPLC-UV chromatograms obtained for a red wine (top) and the SPE extract of the same wine (bottom). Injection volumes were 5 µL and the signal was recorded at 200 nm. Ethyl acetate (solvent) appears at ~ 7 minutes in the chromatogram of the SPE extract. A blank trace was subtracted from both chromatograms to compensate for absorption of acetonitrile at the detection wavelength.

6.3.2. HPLC-MS/MS analysis of EC

Liquid chromatography – tandem mass spectrometry was selected as analytical technique for the determination of EC in alcoholic beverages due to advantages such as high loading capacity, sensitivity and selectivity. Chromatographic separation was performed in NP mode utilising an aminopropyl column. EC is strongly retained under these conditions, affording separation *via* a gradient of acetone in isooctane. Ionisation in the apolar elution solvent was *via* atmospheric pressure chemical ionisation (APCI), which utilises gas-phase ionisation and is inherently suited for use with apolar effluents. In contrast, electrospray ionisation (ESI), which employs liquid phase ionisation, did not provide efficient ionisation under these conditions [25,26].

Figure 6.2 shows typical MRM chromatograms obtained for the analysis of an EC standard and red wine. The combination of RP-SPE, gradient NP separation and MS/MS detection rendered the method free from sample matrix interferences. Moreover, the high loadability inherent to LC, in combination with MRM detection, provides good sensitivity (see validation results further). The intrinsic injection precision of LC as well as the absence of matrix effects also obviated the requirement of an internal standard to normalise sample introduction.

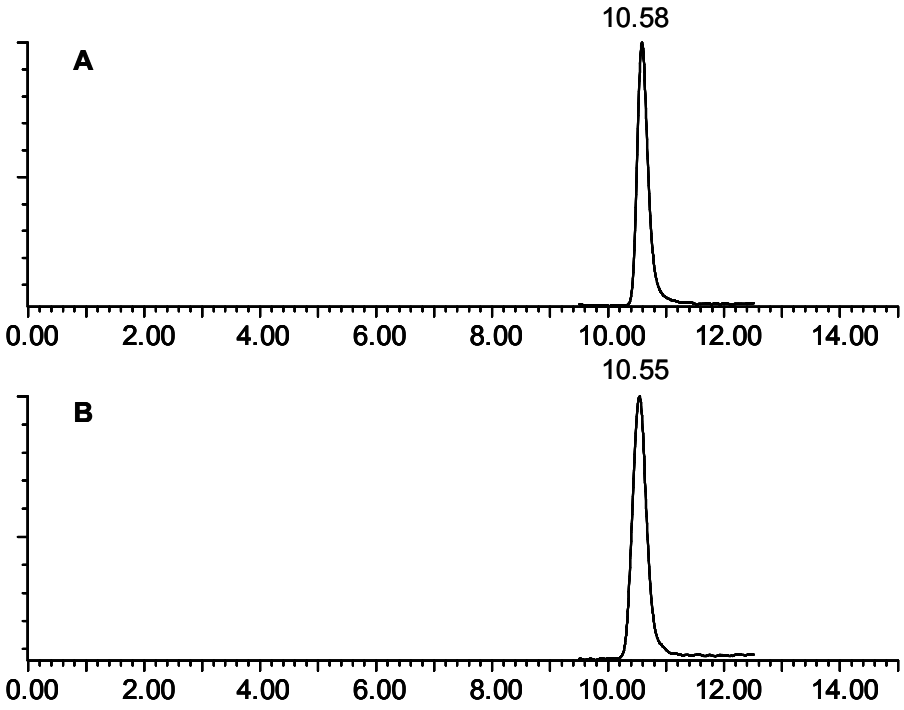


Figure 6.2. MRM Chromatograms of (A) a standard of 10.1 µg/L EC and (B) the extract of a red wine sample containing 11.9 µg/L EC.

As the developed SPE procedure yields extracts in ethyl acetate/isooctane, this sample preparation strategy may also be used in combination with GC analysis. Since elaborate sample preparation procedures are often required for GC methods (especially for wine samples), this procedure may find universal application for methods of EC determination. Preliminary experiments with GC-MS in SIM mode (results not shown) revealed that these extracts are fully compatible with GC, although the sensitivity and selectivity of a quadrupole detector is insufficient for accurate quantitation without prior pre-concentration. However, GC-MS/MS or GCxGC-MS may provide sufficient selectivity to be used successfully in combination with the simple SPE procedure reported here.

6.3.3. Validation of the optimised SPE-NP-LC-MS/MS method

A linear response (r^2 = 0.9999) was obtained between 51 pg and 101 380 pg on column. The limits of detection (LOD) and quantitation (LOQ) were determined as the smallest amount of analyte that produce a signal that is three and ten times the average noise level, respectively. The LOD and LOQ were 25 and 83 pg EC on column, respectively. For the different categories of products analysed these amounts correspond to sample concentrations given in Table 6.1, taking into consideration a maximum injection volume of 100 µL and the fact that sample pre-concentration was matrix dependent. The accuracy of the method was assessed as the recovery from samples fortified with EC prior to sample preparation. A total of 55 recovery measurements was performed at three levels (1, 10 and 100 µg/L), and the results are summarised in Table 6.2. The average recovery of the method across all sample matrices (*n* = 55) was 94.5% (RSD = 8.4%). While this performance is adequate for quantitative purposes, further improvement may also be obtained by using isotopically labelled standards for quantitation.

It should be noted that although validation was performed based on South African wine and spirit samples, the same methodology is expected to be equally applicable to a wide range of similar products. Samples containing much higher concentrations than those reported here (for example cachaças) may then be diluted so that concentrations fall within the linear range of the method, in this manner further reducing the risk of matrix interferences (which were not observed for the samples analysed here).

Table 6.1. Minimum detectable and quantifiable limits for different categories of products obtained by SPE-LC-MS/MS.

Sample type	Concentration factor	LOD (µg/L)	LOQ (µg/L)
Natural wine (<15% alc.)	1	0.25	0.83
Fortified wine (<20% alc.)	0.8	0.31	1.0
Spirits (<45% alc.)	0.4	0.63	2.1

Table 6.2. Summary of results obtained for recovery experiments involving EC in white, red and fortified wines, and spirits.

	White wine, *n* = 4	Red wine, *n* = 6	Fortified wine, *n* = 5	Spirits, *n* = 5
1 µg/L				
average (%)	92.8	90.9	104.6	
RSD (%)	6.9	7.1	7.6	
10 µg/L				
average (%)	99.1	90.7	104.7	88.4
RSD (%)	5.7	6.5	4.7	7.2
100 µg/L				
average (%)	98.3	88.8	92.6	92.2
RSD (%)	7.0	6.5	5.9	2.4
Overall recovery	**All wines, *n* = 30**		**Fortified wine, *n* = 15**	**Spirits, *n* = 10**
s (%)	6.9		8.2	4.9
average (%)	92.8		100.6	90.3
RSD (%)	7.4		8.2	5.5

The reproducibility of the LC-MS/MS system expressed as the standard deviation of the peak area obtained for a standard was 5.5% (*n* = 8). Intra-assay precision, assessed as the repeatability of the recovery, was 8.4% at concentrations of 1, 10 and 100 µg/L for wines and fortified wine, and 10 and 100 µg/L for spirits.

Compound identification was based on retention time and a single ion transition, *m/z* = 90.1 (M + 1) ® 62.3. One secondary ion transition (*m/z* = 90.1 ® 44.5) was observed, but the intensity was too weak to be detected consistently at the typical analyte concentrations (the ratio of these transitions is 35:1, therefore the secondary transition was evident at concentrations of ~30 µg/L and higher). Quantitative results for three red wines obtained by the SPE-NP-LC-MS/MS method were also compared with results obtained independently using the official OIV method [21] and results for this limited sample set showed good agreement (r^2 = 0.95).

6.3.4. Survey of the ethyl carbamate content of South African wines and spirits

Previous studies have shown that varying amounts of EC are present in different alcoholic beverages. The European Food Safety Authority has reported over 33 000 testing results for alcoholic beverages. Median EC concentrations of up to 5 µg/L for beer and wine, 21 µg/L for spirits other than fruit brandy, and 260 µg/L for fruit brandy were obtained [9]. In other studies, the following concentrations of EC were reported (sample numbers in parenthesis); whiskies

(18): <5-206 µg/L, port (4): 14-21 µg/L, liqueur (8): 9-439 µg/L, sherry (12): <5-60 µg/L and wine (31): 1-18 µg/L [17]. Old and new Spanish and American fortified wines contained concentrations varying between <0.1 and 1256 µg/L [16], while 20 fortified Madeira wines (dry to sweet) contained EC concentrations ranging from 54.1 to 162.5 µg/L [6]. Concentrations of between 55 µg/L and 700 µg/L (average 221 µg/L) were reported for some commercial Brazilian cachaças [27]. Distilled spirits are particularly susceptible to EC contamination: Weber and Sharypov reported a concentration rage of 10-12 000 µg/L, depending on the origin of the spirit [7]. South African wines entered in the 2001 Veritas show generally displayed low levels of EC; red wines of all cultivars contained an average of 7.30 µg/L EC (*n* = 687), and white wines (all cultivars, *n* = 442) contained an average of 1.74 µg/L EC [12].

In view of the risk of contamination inherent to alcoholic beverages, the developed method was applied to provide an overview of EC concentrations for a range of South African commodities. The EC content of three broad categories of South African alcoholic beverages was determined, namely wine, fortified wine and spirits. A small number of imported wine (4) and spirit samples (6) were also analysed for comparison. The results are presented in full in the Supplementary information, Tables S6.1 to S6.4. Table 6.3 presents a concise summary of data for the main categories of alcoholic beverages analysed. EC levels displayed considerable variance across these products, with concentrations ranging from 1.8 to 95 µg/L (RSD = 100%) in 166 South African commodities.

Table 6.3. Summary of the EC content (µg/L) of South African commodities. For complete data, see the Supplementary information, Tables S6.1-S6.4.

Product	*n*	age (years)	min	max	average	RSD (%)
Dry white wine	34	1 - 6	1.8	23	7.8	85
Dry red wine	72	1 - 9	2.4	31	11	62
Fortified wine	21	2 - 34	2.8	79	26	89
Brandy	26	3 - 20	4.4	95	19	105

6.3.4.1. Wine

The EC content of 106 South African wine samples of vintages between 2000 and 2008 varied between 1.8 and 31 µg/L (RSD = 69%). White wines contained consistently lower concentrations compared to red wines of the same vintages. This phenomenon was also noted by other authors and has been ascribed to the fact that red wines contain higher levels of EC forming precursors [12]. A clear relationship ($p = 0.01$ in each case) was evident between the EC content of red and white wines and age (Figure 6.3A and B).

Waldner and Augustyn reported that Pinotage juice consistently contains relatively high concentrations of α-amino nitrogen compared to other red cultivars [12]. Although insufficient data were available in this study to attempt statistical discrimination of cultivars based upon EC content, Pinotage wines did, on average, contain higher EC levels compared to other red wine cultivars. Wines of the 2007 vintage analysed in this study showed that Pinotage (*n* = 11) contained an average of 11 µg/L EC, while other red wines (*n* = 10) averaged 5.0 µg/L. The EC content of four imported wines (two white and two red, aged 1-3 years) ranged from 1.0 to 6.5 µg/L. These levels are comparable to those of South African wines of the same age.

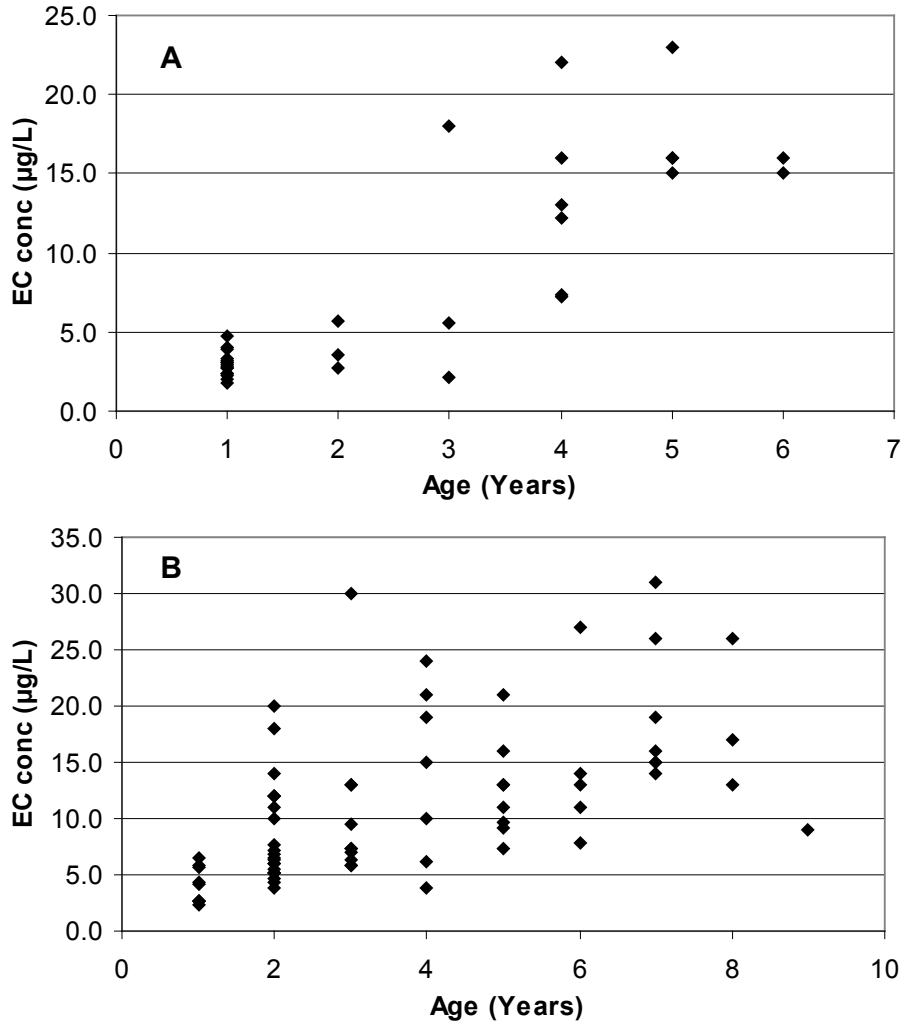


Figure 6.3. EC content of (A) white and (B) red wines as a function of age at the time of analysis. The linear correlation coefficients (r^2) for these data are (A) 0.72 and (B) 0.33, respectively.

6.3.4.2. Fortified wines

The EC content of South African fortified wines ranged from 2.8 to 79 µg/L (RSD = 90%) in 25 samples. As in wine, the EC content increases with age (p = 0.01, Figure 6.4A). Note that the oldest wine investigated in this study, a 1975 vintage fortified wine (aged 34 years), still complied with Canadian and Czech regulations [7,9], which stipulate a maximum EC level of 100 µg/L for this commodity.

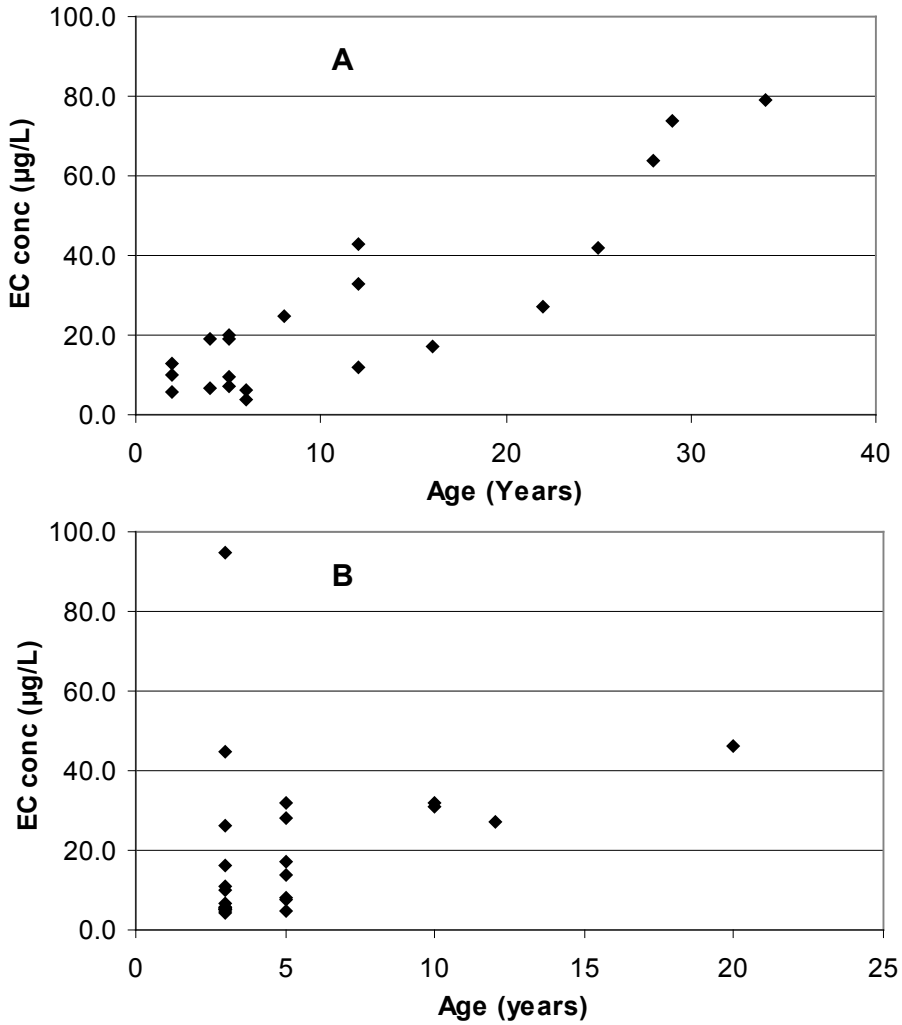


Figure 6.4. EC content of South African fortified wines (A) and brandies (B) as a function of age at the time of analysis. The linear correlation coefficient (r^2) for the fortified wines is 0.79.

6.3.4.3. Distilled spirits

The EC content of South African distilled spirits ranged between 1.9 and 95 µg/L (RSD = 104%) in 35 samples. From a South African perspective, brandy is by far the most important distilled product. The EC content of local brandies (*n* = 26) displayed no clear increase as a function of age ($p > 0.10$, Figure 6.4B). Note that where no maturation information was supplied for brandy samples, the age was taken to be three years, as South African regulations [28] stipulate that local brandies should contain at least 30% pot-still spirit that was matured for a minimum of three years. EC levels in 20-year-old brandy samples were not elevated significantly compared to 3-year-old products. This observation is supported by the finding that the EC content of seven brandy samples showed insignificant (5% average) increases when subjected to reflux boiling for three hours (see further). In contrast, the EC content of South African whisky and rum samples increased (average 37%) when subjected to reflux boiling for three hours. It has been reported that >80% of the EC in distilled spirits is produced during distillation or within 48 hours thereafter [7]. However, post-distillation EC formation in maturing Scotch grain whisky is a known phenomenon and is ascribed to a series of cyanide-based precursors that are present in these grain-based products [7,29]. The average increase in EC concentration upon subjecting three Scotch whiskies to reflux boiling for three hours was 8%. These differences in the rate of EC formation may possibly be ascribed to different mechanistic pathways involved. However, the EC content of distilled spirits is clearly more stable compared to wines, for which significant increases were observed following reflux boiling.

6.3.5. Factors influencing the formation of EC in alcoholic beverages

In the presence of ethanol, various pathways exist for the formation of EC in alcoholic beverages [30,31]. In grain-based spirits, cyanide-containing precursors originating from cereal grains react during fermentation and distillation to produce EC [7,9,29]. The high barley content in bourbon mash combined with traditional bourbon distillation practices makes bourbon particularly susceptible to EC contamination [3].

In wine, residual urea not consumed as yeast nutrient during fermentation is considered the main precursor for the formation of EC. During wine fermentation, amino acids are metabolised by yeast cells, resulting in excretion of urea into the medium [12]. The amount of urea released depends on the yeast strain and on the amino acid content of the juice [16,30]. EC is formed in wine *via* acid-catalysed reaction with ethanol [30,32]. The extent of this reaction during ageing and storage is regulated mainly by temperature, but it also depends on

the urea and ethanol concentrations, and wine pH [7,12,30,32-34]. These formation processes occur continuously during ageing, resulting in the accumulation of EC as a function of time [12,32,34]. Light exposure during ageing and storage is reported to have no significant effect on the EC content of wine [32,34]. In addition, EC may be hydrolysed to carbamic acid, which in turn may decompose to release ammonia and carbon dioxide [34].

The EC content of alcoholic beverages at any given time is therefore determined by the complex interaction of various factors and may be the sum of two opposing processes, formation on the one hand and hydrolytic degradation on the other. These diverse factors also possibly account for the widely varying rates of formation of EC reported to occur in wines. For example, Tegmo-Larsson and Spittler reported increases in wine EC concentration of between zero and 30 fold for wines kept at 43ºC for 12 months [34]. A number of experiments were therefore performed in order to shed some light on the formation of EC in alcoholic beverages.

The effect of alcohol content on the formation of EC in a wine sample was investigated by subjecting a series of sub-samples, with a range of alcohol concentrations (but constant wine matrix), to reflux boiling for three hours. The EC content of these samples was determined after suitable dilution to compensate for the increased alcohol content. As is evident from Figure 6.5 (x1 vs. y1 axis), the formation of EC was accelerated with increasing alcohol content, such that approximately 10% more EC was produced in the same time at an alcohol content of 31.2%(v/v) compared to 11.2%(v/v). In the range of typical wine alcohol concentrations (~10-15%), relatively minor variations in the EC concentration may, however, be expected to result from the involvement of ethanol alone.

The rate of EC formation as a function of wine pH was investigated by subjecting a series of pH adjusted wine samples to reflux boiling for two hours. The pH of a red wine (pH 3.65) was adjusted to yield wine samples with pH values ranging from 3.01 to 5.33. From Figure 6.5 (x2 vs. y2 axis) it is evident that EC formation in these wine matrices is accelerated at low pH, although once again the expected effect should be minimal within the typical wine pH range (~3-4). Stevens and Ough reported that EC formation in wines is the result of an acid-catalysed ethanolysis reaction with urea and citruline [32].

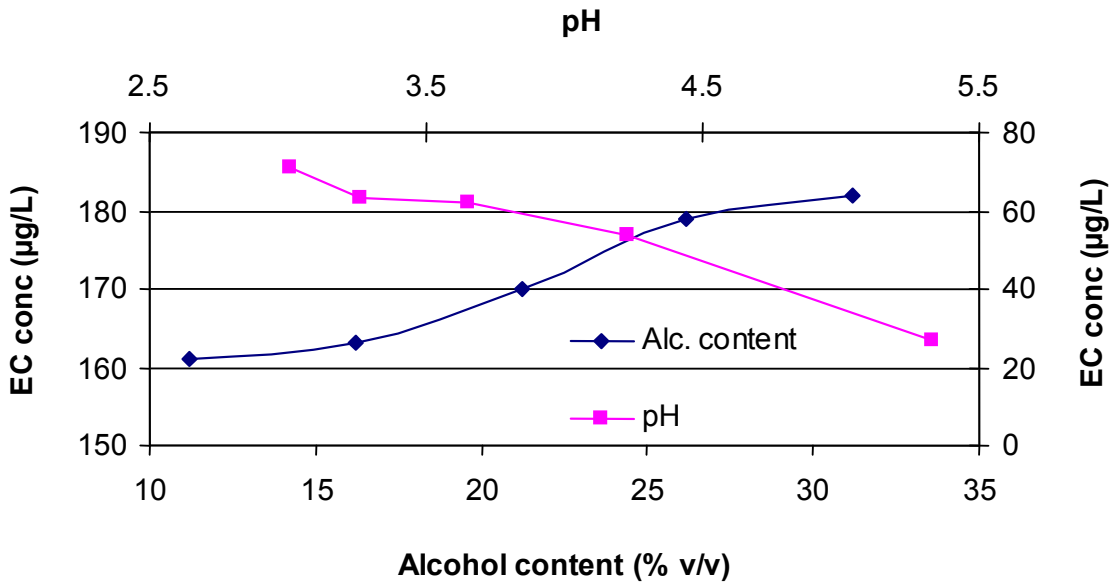


Figure 6.5. The EC content of a Pinotage wine fortified to produce a series of samples with increasing alcohol content and subjected to reflux boiling for three hours (x1 vs. y1), and EC content of a series of pH adjusted Pinotage wine samples subjected to reflux boiling for two hours (x2 vs. y2).

The EC concentration of a wine sample increased as a function of time (approximately linearly, from 20 µg/L to 330 µg/L) when subjected to reflux boiling for five hours. The potential EC concentration of this wine is therefore in excess of 330 µg/L. However, the potential for EC accumulation may vary significantly between wines [34]. We found that four Pinotage wines subjected to reflux boiling for three hours displayed increases in the EC content by factors of 4, 10 12 and 20, respectively. This variation (within the same cultivar) demonstrates differences in the rate of EC formation between wines, most likely due to corresponding differences in the concentrations of precursors (especially urea) and other variables, such as pH and alcohol content. Stevens and Ough reported that the concentrations of urea in wine may vary between 1 and 10 mg/L, which suggests that relatively high amounts of EC may accumulate if only a fraction of urea is converted to EC [32].

The EC content of brandies is more stable than that of wine, as evidenced by the fact that only marginal increases are noted upon reflux boiling of brandy samples. The fact that EC is volatile suggests that it enters brandy during distillation [7]. This was confirmed by distillation of a Chenin blanc wine sample followed by analysis of the distillate and the wine residue. The EC content of the original wine was 1.8 µg/L, while the EC content of the distillate (distilled to 50% of the initial volume) was 2.2 µg/L. The remaining wine left over from the distillation process contained 5.6 µg/L EC. The EC content of the same wine subjected to

reflux boiling for one hour was 13 µg/L. That the distillation residue contained lower concentrations of EC compared to the same wine subjected to reflux boiling may possibly be due to the (continuous) removal of ethanol during distillation that retards the formation reaction relative to reflux conditions (where the ethanol concentration remains constant).

6.4. Conclusions

EC is universally present in fermented alcoholic beverages such as wine and fortified wine and in distilled spirits such as whisky and brandy [2,3,7]. In light of its possible carcinogenicity [5,11] it is imperative that robust and simple analytical methods are available to asses the extent of EC contamination, especially in view of the fact that EC levels are regulated in several countries. This study describes a novel SPE procedure that is significantly simpler than published sample preparation methods, yields quantitative recovery of the analyte, requires minimal sample volume and offers reduced solvent consumption. Moreover, the solvents used (ethyl acetate and isooctane) are considerably less toxic than dichloromethane, which is used, for example, in the official OIV method. The extracts yielded by the described sample preparation strategy are compatible with GC techniques, but was validated and applied here in combination with LC-MS/MS. The performance of the overall SPE-LC-MS/MS method was such that EC could be quantified in all samples analysed, including wines, fortified wines and spirits, using external standard quantification.

The method was successfully applied to study the occurrence of EC in South African alcoholic beverages. The EC content of South African wines displays a clear correlation with vintage – a phenomenon that is evident for white, red as well as fortified wines. Despite this relationship with sample age, the majority of products investigated contained minor levels of EC. EC concentrations in South African brandy samples were found to be more stable with respect to age. Since all samples in this study are commercial products, no information regarding production parameters and storage conditions are available – a factor that certainly contributed to the variation that was observed in the datasets. However, EC levels found in South African products are generally much lower than those currently enforced internationally.

Investigation of the factors responsible for variations observed in EC levels demonstrated that the potential for EC formation varies significantly between wines (even within cultivars) and that temperature, alcohol content and pH affect the rate of EC formation in wine. The variable that has the greatest effect upon EC formation is temperature – an observation supported by the findings of earlier studies [12,32,34].

References

[1] S. Budavari, The Merck Index: An encyclopedia of chemicals, drugs and biologicals, Merck & Co. Inc., Rahway, U.S.A. (1989).
[2] R.S. Jackson, Wine science: Principles, practice and perception, Academic Press, San Diego, U.S.A. (2000).
[3] K. Jacques, T.P. Lyons, D.R. Kelsall, The alcohol textbook, Nottingham University Press, Thrumpton, U. K. (1999).
[4] JECFA monograph, Sixty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives, WHO and FAO, (2006). Cited February 2011, Available from: http://whqlibdoc.who.int/publications/2006/9241660554_eng.pdf
[5] IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 96, Alcoholic beverage consumption and ethyl carbamate (urethane), Lyon, France: in press.
[6] R. Perestrelo, S. Petronilho, J.S. Câmara, S.M. Rocha, J. Chromatogr. A (2010) doi:10.1016/j.chroma.2010.03.027. 2010.
[7] J.V. Weber, V.I. Sharypov, Environ. Chem. Lett. 7 (2009) 233.
[8] Codex draft, Joint FAO/WHO food standards programme, CODEX committee on contaminants in foods, third session. Codex alimentarius commission E (2009), Available from: ftp://ftp.fao.org/codex/cccf3/cf03_13e.pdf
[9] The European Food Safety Authority Journal 551 (2007) 1.
[10] D.W. Lachenmeier, B. Schehl, T. Kuballa, W. Frank, T. Senn, Food Add. Cont. 22 (2005) 397.
[11] D.W. Lachenmeier, M.C.P. Lima, I.C.C. Nóbrega, J.A.P. Pereira, F. Kerr-Corrêa, F. Kanteres, J. Rehm, BMC Cancer 10 (2010) 266.
[12] M. Waldner, O.P.H. Augustyn, Wineland (2005) 98.
[13] C. Fauhl, R. Wittkowski, J. High Res. Chrom. 15 (1992) 203.
[14] M.A. Ferreira, J.O. Fernandes, Am. J. Enol. Vitic. 43 (1992) 339.
[15] B.J. Canas, F.L. Joe, G.W. Diachenko, G. Burns, J. AOAC Int. 77 (1994) 1530.
[16] C.E. Daudt, C.S. Ough, D. Stevens, T. Herraiz, Am. J. Enol. Vitic. 43 (1992) 318.
[17] M.J. Dennis, N. Howarth, P.E. Key, M. Pointer, R.C. Massey, Food Add. Cont. 6 (1989) 383.
[18] E. Jagerdeo, S. Dugar, G.D. Foster, H. Schenk, J. Agric. Food Chem. 50 (2002) 5797.
[19] R.S. Whiton, B.W. Zoecklein, Am. J. Enol. Vitic. 53 (2002) 60.
[20] D.W. Lachenmeier, U. Nerlich, T. Kuballa, J. Chromatogr. A 1108 (2006) 116.
[21] Compendium of international methods of wine and must analysis. Office International de la Vigne et du Vin (OIV), Paris, France (2002).
[22] P. Herbert, L. Santos, M. Bastos, P. Barros, A. Alves, J. Food Sci. 67 (2002) 1616.
[23] S-K. Park, C.T. Kim, J-W. Lee, O.H. Jhee, A.S. Om, J.S. Kang, T.W. Moon, Food Control 18 (2007) 975.
[24] A. Mirzoian, A. Mabud, J. AOAC Int. 89 (2006) 1048.
[25] R. Kostiainen, T.J. Kauppila, J. Chromatogr. A 1216 (2009) 685.
[26] M.C. McMaster, LC/MS a practical user's guide, John Wiley & Sons Inc., New Jersey, U.S.A. (2005).
[27] I.C.C. Nóbrega, J.A.P. Pereira, J.E. Paiva, D.W. Lachenmeier, Food Chem. 117 (2009) 639.
[28] Liquor Products Act, Government Gazette of the Republic of South Africa, Act 60 (1989).
[29] W.M. MacKenzie, A.H. Clyne, L.S. MacDonald, J. Inst. Brew. 96 (1990) 223.
[30] C.S. Ough, D. Stevens, T. Sendovski, Z. Huang, D. An, Am. J. Enol. Vitic. 41 (1990) 68.
[31] B. Zimmerli, J. Schlatter, Mutation Research 259 (1991) 325.
[32] D.F. Stevens, C.S. Ough, Am. J. Enol. Vitic. 44 (1993) 309.
[33] I.M. Tegmo-Larsson, T. Henick-Kling, Am. J. Enol. Vitic. 41 (1990) 269.
[34] I.M. Tegmo-Larsson, T.D. Spittler, Food Sci. 55 (1990) 1166.

Supplementary information

Table S6.1. EC data for South African natural wines.

No.	Type	Origin	Cultivar	Vintage	Age (years)	EC (µg/L)
1	Red Wine, Dry	Robertson	Shiraz	2007	2	12
2	Red Wine, Dry	Western Cape	Cabernet Sauvignon	2007	2	4.3
3	Red Wine, Dry	Western Cape	Pinotage	2007	2	6.0
4	Red Wine, Dry	Stellenbosch	Cabernet Sauvignon	2006	3	7.3
5	Red Wine, Dry	Paarl	Pinotage	2007	2	10
6	Red Wine, Dry	Walker Bay	Pinotage	2007	2	5.2
7	White Wine, Dry	Coastal Region	Chardonnay	2008	1	3.1
8	White Wine, Dry	Paarl	Chardonnay	2008	1	2.4
9	White Wine, Dry	Slanghoek	Chenin blanc	2008	1	2.9
10	White Wine, Dry	Paarl	Chenin blanc	2008	1	4.0
11	White Wine, Dry	Franschhoek	Semillon	2007	2	2.7
12	White Wine, Dry	Robertson	Sauvignon blanc	2008	1	2.2
13	Red Wine, Dry	Western Cape	Cabernet Sauvignon	2008	1	2.7
14	Red Wine, Dry	Robertson	Ruby Cabernet	2008	1	2.7
15	Red Wine, Dry	Stellenbosch	Pinotage	2007	2	7.6
16	Red Wine, Dry	Coastal Region	Shiraz	2007	2	4.6
17	Red Wine, Dry	Paarl	Shiraz	2008	1	2.4
18	Red Wine, Dry	Paarl	Cabernet Sauvignon	2008	1	2.6
19	Red Wine, Dry	Coastal Region	Shiraz	2002	7	31
20	Red Wine, Dry	Western Cape	Shiraz	2008	1	6.5
21	Red Wine, Dry	Paarl	Cabernet Sauvignon	2005	4	10
22	Red Wine, Dry	Paarl	Shiraz	2005	4	3.8
23	Red Wine, Dry	Coastal Region	Pinotage	2007	2	12
24	Red Wine, Dry	Coastal Region	Merlot	2005	4	19
25	Red Wine, Dry	Paarl	Pinotage	2008	1	5.8
26	White Wine, Dry	Hemel-en-Aarde valley	Chardonnay	2008	1	2.7
27	Red Wine, Dry	Stellenbosch	Pinotage	2006	3	13
28	White Wine, Dry	Walker Bay	Chenin blanc	2008	1	3.0
29	White Wine, Dry	Paarl	Chenin blanc	2008	1	3.3
30	White Wine, Dry	Paarl	Chenin blanc	2006	3	2.1
31	White Wine, Dry	Stellenbosch	Sauvignon blanc	2003	6	16
32	White Wine, Dry	Durbanville	Sauvignon blanc	2004	5	16
33	White Wine, Dry	Western Cape	Sauvignon blanc	2005	4	7.2
34	White Wine, Dry	Robertson	Sauvignon blanc	2004	5	23
35	White Wine, Dry	Coastal Region	Sauvignon blanc	2004	5	15
36	White Wine, Dry	Durbanville	Sauvignon blanc	2005	4	13
37	Red Wine, Dry	Stellenbosch	Cabernet Sauvignon	2006	3	6.4
38	Red Wine, Dry	Robertson	Pinotage	2007	2	5.1
39	Red Wine, Dry	Paarl	Merlot	2007	2	6.5
40	Red Wine, Dry	Stellenbosch	Merlot	2006	3	5.8
41	Red Wine, Dry	Western Cape	Shiraz	2005	4	6.1
42	Red Wine, Dry	Western Cape	Cabernet Sauvignon	2007	2	6.8
43	Red Wine, Dry	Stellenbosch	Cabernet Sauvignon	2006	3	5.8
44	White Wine, Dry	Coastal Region	Chardonnay	2008	2	3.9
45	White Wine, Dry	Western Cape	Chardonnay	2005	4	12
46	White Wine, Dry	Stellenbosch	Sauvignon blanc	2008	1	2.7
47	Red Wine, Dry	Stellenbosch	Shiraz	2004	5	21
60	Red Wine, Dry	Coastal Region	Pinotage	2007	2	20
61	Red Wine, Dry	Robertson	Shiraz	2000	9	9.0
62	Red Wine, Dry	Wellington	Shiraz	2008	1	5.6

63	Red Wine, Dry	Western Cape	Merlot	2008	1	4.2
64	Red Wine, Dry	Western Cape	Cabernet Sauvignon	2008	1	4.3
65	Red Wine, Dry	Paarl	Pinotage	2006	3	13
68	White Wine, Dry	Western Cape	Chardonnay	2008	1	3.1
69	Red Wine, Dry	Coastal Region	Pinotage	2007	2	12
70	Red Wine, Dry	Paarl	Merlot	2007	2	5.5
71	Red Wine, Dry	Coastal Region	Shiraz	2007	2	5.2
72	Red Wine, Dry	Western Cape	Pinotage	2007	2	14
73	Red Wine, Dry	Stellenbosch	Pinotage	2004	5	9.7
74	Red Wine, Dry	Western Cape	Pinotage	2008	1	7.1
75	Red Wine, Dry	Coastal Region	Shiraz	2006	3	7.0
76	Red Wine, Dry	Franschhoek	Cabernet Sauvignon	2006	3	7.3
77	White Wine, Dry	Western Cape	Sauvignon blanc	2008	1	3.2
78	White Wine, Dry	Western Cape	Chardonnay	2008	1	4.7
79	Red Wine, Dry	Western Cape	Cabernet Sauvignon	2007	2	6.0
80	Red Wine, Dry	Western Cape	Cabernet Sauvignon	2007	2	3.8
81	Red Wine, Dry	Western Cape	Cabernet Sauvignon	2007	2	6.3
82	Red Wine, Dry	Western Cape	Pinotage	2005	4	21
83	White Wine, Dry	Western Cape	Chardonnay	2008	1	2.4
84	White Wine, Dry	Stellenbosch	Sauvignon blanc	2003	6	15
85	White Wine, Dry	Western Cape	Sauvignon blanc	2005	4	7.3
86	White Wine, Dry	Goudini	Colombard	2005	4	16
87	White Wine, Dry	Western Cape	Sauvignon blanc	2005	4	22
88	Red Wine, Dry	Tulbagh	Shiraz	2004	5	16
89	Red Wine, Dry	Stellenbosch	Pinotage	2007	2	11
93	Red Wine, Dry	Stellenbosch	Shiraz	2004	5	13
94	White Wine, Dry	Coastal Region	Chenin blanc	2008	1	2.0
95	White Wine, Dry	Stellenbosch	Chenin blanc	2008	1	1.8
97	Red Wine, Dry	Coastal Region	Merlot	2004	5	13
98	Red Wine, Dry	Stellenbosch	Cabernet Sauvignon	2001	8	13
99	Red Wine, Dry	Western Cape	Shiraz	2006	3	9.5
100	Red Wine, Dry	Western Cape	Pinotage	2007	2	18
101	Red Wine, Dry	Western Cape	Pinotage	2006	3	30
103	Red Wine, Dry	Stellenbosch	ns [a]	2001	8	26
104	Red Wine, Dry	Simonsberg	Cabernet Sauvignon	2002	7	15
105	Red Wine, Dry	Jonkershoekvalley	ns	2002	7	14
106	Red Wine, Dry	Swartland	Shiraz	2002	7	19
107	Red Wine, Dry	Hemel-en-Aarde valley	Cabernet Sauvignon	2002	7	16

108	Red Wine, Dry	Paarl	Pinotage	2003	6	27
109	Red Wine, Dry	Stellenbosch	Shiraz	2003	6	13
110	Red Wine, Dry	Stellenbosch	Shiraz	2003	6	7.9
111	Red Wine, Dry	Coastal Region	Cabernet Sauvignon	2001	8	17
112	Red Wine, Dry	Wellington	Cabernet Sauvignon	2003	6	11
113	Red Wine, Dry	Stellenbosch	Shiraz	2004	5	7.4
114	Red Wine, Dry	Stellenbosch	Merlot	2004	5	9.1
115	Red Wine, Dry	Robertson	Shiraz	2004	5	11
116	Red Wine, Dry	Western Cape	Pinotage	2005	4	24
117	Red Wine, Dry	Western Cape	Cabernet Sauvignon	2005	4	15
118	White Wine, Dry	Elgin	Sauvignon blanc	2004	5	16
119	White Wine, Dry	Stellenbosch	Chardonnay	2006	3	18
120	White Wine, Dry	Robertson	Chardonnay	2006	3	5.6
121	White Wine, Dry	Robertson	Chardonnay	2007	2	5.7
122	White Wine, Dry	Stellenbosch	Chardonnay	2007	2	3.6
123	Red Wine, Dry	Stellenbosch	Shiraz	2002	7	15
124	Red Wine, Dry	Paarl	Pinotage	2002	7	26
125	Red Wine, Dry	Paarl	Shiraz	2003	6	14

[a] Not specified.

Table S6.2. EC data for South African fortified wines.

No.	Type	Origin	Vintage	Age (years)	EC (µg/L)
48	Fortified Wine	Coastal Region	2001	8	25
49	Fortified Wine	ns [a]	1993	16	17
50	Fortified Wine	Paarl	1997	12	33
51	Fortified Wine	Stellenbosch	1987	22	27
52	Fortified Wine	Boberg	1981	28	64
53	Fortified Wine	Boberg	1984	25	42
54	Fortified Wine	Paarl	1980	29	74
55	Fortified Wine	Paarl	2004	5	20
56	Fortified Wine	ns	ns	-	16
57	Fortified Wine	Tulbagh	2005	4	6.5
58	Fortified Wine	Western Cape	ns	-	15
59	Fortified Wine	Western Cape	ns	-	21
66	Fortified Wine	Robertson	2004	5	9.6
67	Fortified Wine	Robertson	2003	6	6.1
90	Fortified Wine	Stellenbosch	2004	5	19
91	Fortified Wine	ns	ns	-	2.8
92	Fortified Wine	Stellenbosch	2007	2	5.9
96	Fortified Wine	Robertson	1975	34	79
126	Fortified Wine	Western Cape	2005	4	19
127	Fortified Wine	Paarl	1997	12	43
128	Fortified Wine	Coastal Region	2007	2	13
129	Fortified Wine	Stellenbosch	2003	6	3.8
130	Fortified Wine	Stellenbosch	1997	12	12
131	Fortified Wine	Robertson	2004	5	7.0
132	Fortified Wine	Calitzdorp	2007	2	9.9

[a] Not specified.

Table S6.3. EC data for South African spirits.

No.	Type	Aged	EC (µg/L)
S1	Brandy	3	5.1
S2	Brandy	20	46
S3	Brandy	10	32
S4	Whisky	3	13
S5	Brandy	3	4.4
S6	Brandy	3	45
S7	Brandy	3	5.4
S8	Whisky	3	11
S9	Whisky	3	6.1
S10	Brandy	3	5.5
S11	Brandy	2	4.8
S12	Brandy	12	27
S13	Rum	-	2.7
S14	Brandy	3	5.7
S15	Brandy	3	5.2
S16	Brandy	5	5.0
S17	Brandy	3	26
S18	Brandy	3	16
S19	Brandy	3	95
S20	Brandy	5	28
S21	Brandy	3	11
S22	Brandy	3	9.8
S23	Brandy	3	5.3
S24	Brandy	5	14
S25	Brandy	10	31
S26	Brandy	3	6.8
S27	Whisky	5	20
S28	Brandy	5	17
S29	Brandy	5	7.5
S30	Brandy	5	32
S31	Husk brandy	-	1.9
S32	Unspecified spirit	-	22
S33	Unspecified spirit	-	10
S34	Unspecified spirit	-	17
S41	Brandy	5	7.9

Table S6.4. EC data for imported products.

No.	Type	Origin	Vintage	Age (years)	EC (µg/L)
S35	Scotch Whisky	Scotland	-	12	20
S36	Scotch Whisky	Scotland	-	18	60
S37	Scotch Whisky	Scotland	-	-	11
S38	Scotch Whisky	Scotland	-	-	7.5
S39	Scotch Whisky	Scotland	-	-	17
S40	Bourbon	U.S.A.	-	-	110
I1	Red Wine, Dry	Israel	2006	3	6.5
I2	White Wine, Dry	Israel	2007	2	2.0
I3	Red Wine, Dry	Chile	2007	2	3.9
I4	White Wine, Dry	Chile	2008	1	1.0

Chapter 7

Quantitative survey of 3-alkyl-2-methoxypyrazines and first confirmation of 3-ethyl-2-methoxypyrazine in South African Sauvignon blanc wines *

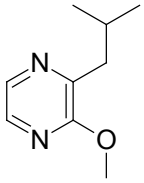
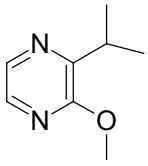
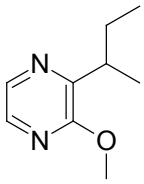
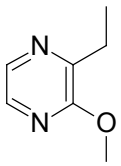
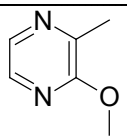
* Submitted for publication to S. Afr. J. Enol. Vitic.

7.1. Introduction

Sauvignon blanc is a distinctive dry white wine with a characteristic aroma described as including vegetative, herbaceous and green pepper nuances. Three 3-alkyl-2-methoxypyrazines (MPs) namely, 3-isobutyl- (IBMP), 3-*sec*-butyl- (SBMP) and 3-isopropyl-2-methoxypyrazine (IPMP), are principally responsible for the distinctive vegetal character of the cultivar [1-7]. These grape-derived flavour components have extremely low sensory detection thresholds (Table 7.1). Some congeners influence the aroma of white wine at concentrations of 1-2 ng/L, while their detection threshold in red wine is 10-15 ng/L [2,5,6]. In wine, MPs typically occur in trace level amounts, with a combined concentration that is in the order of 1-40 ng/L [2].

The most abundant congener found in Sauvignon blanc wine is invariably IBMP, representing approximately 80% of the total, while IPMP and SBMP occur in roughly equal amounts [5,8,9]. The occurrence of 3-ethyl-2-methoxypyrazine (EMP) in wine has also been reported [1], while the possible occurrence of 3-methyl-2-methoxypyrazine (MMP) has been suggested based on a feasible biosynthetic route [2,3,7,10]. These latter pyrazines are associated with 'earthy' aromas and have relatively high olfactory detection thresholds (Table 7.1); they are therefore unlikely to contribute significantly to the typical 'green' Sauvignon blanc wine aroma. Sala *et al*. [7,10] reported the presence of EMP in Merlot and Cabernet Sauvignon musts and wines, but found concentrations that were consistently below the level of quantification of their methods (0.5-4 ng/L). In a preliminary investigation, Allen *et al*. (1998) reported levels of EMP as high as 1 000 ng/L in a Cabernet Sauvignon wine as well as more than 100 ng/L in a Pinot noir wine [2]. However, there is limited information available pertaining to the occurrence of this compound in grapes and wines of different cultivars. In fact, apart from the single tentative report by Allen *et al.* [2], who found unusually high levels of EMP, no quantitative data on EMP have been reported. This lack of information is largely due to the challenges associated with the analysis of these trace level compounds in the complex wine matrix [11].

Table 7.1. Chemical structures, odour thresholds and flavour properties of the principal 3-alkyl-2-methoxypyrazines found in wine.

Compound	structure	Odour threshold in water (ng/L)[a]	Flavour description[b]
IBMP		1-2	Bell peppers
IPMP		1-2	Bell peppers, green peas
SBMP		1-2	Galbanum, ivy leaves, green peas
EMP		425	Raw potato
MMP		4 000	Roasted peanuts

[a] [2,12]

[b] [12,13]

In grapes, the concentrations of MPs are strongly affected by climatic and viticultural conditions, and generally decrease after *véraison* [2,5,6]. As a consequence, Sauvignon blanc wine possessing the typical green pepper or vegetative aroma is sensitive to production factors such as geographical origin [3,14], mesoclimatic conditions [3,14,15], canopy microclimate [14,16], water availability [16], solar radiation [17,18], time of harvesting [19], viticultural practices [14,20] and the yeast strain used [21]. Marais *et al.* reported that canopy microclimate plays a particularly important role in determining grape MP levels and that exposure to solar radiation, as manipulated by canopy management, has a more prominent effect than climatic temperature alone [14]. Moreover, possible infestations with lady beetles such as *Harmonia axyridis* may also contribute MPs to affected wines. In particular, elevated levels of IPMP and SBMP are

associated with this phenomenon [22]. It has also been reported that bottle closure and packaging type affect MP concentrations during wine storage, with the three major MPs affected to differing degrees. Some closure and packaging types even led to increases in wine IPMP and SBMP concentrations [23]. This sensitivity of MP concentrations to oenological, production and ageing variables possibly contributes to the observed variance in their occurrence in Sauvignon blanc wines.

A previous survey on the occurrence of MPs in South African Sauvignon blanc wines reported concentrations of IBMP ranging from 0.40 to 44 ng/L (RSD of 102%) in 577 wines. Concentrations of IPMP and SBMP ranged from <0.03 to 3.9 ng/L and <0.03 to 3.2 ng/L, respectively [8]. In the current study, an additional 304 Sauvignon blanc wines were analysed and added to the dataset. Data representing a wider span of vintages and origins were obtained, with the aim of improving the statistical representation of the South African Sauvignon blanc industry, in order to allow closer investigation of trends in the data. Malic acid levels were also determined for a subset of samples, since malic acid concentration is strongly affected by viniviticultural variables and can therefore reveal information pertaining to the climate of cultivation and maturity of grapes. Malic acid concentration was used together with general wine parameters such as pH, alcohol content, total acidity (TA) and volatile acidity (VA), in an attempt to identify the factors that are responsible for the production of varietal-typical Sauvignon blanc wines possessing the vegetative aroma.

Moreover, given the limited information on MMP and EMP concentrations in Sauvignon blanc wines, the liquid chromatography – tandem mass spectrometry (LC-MS/MS) method reported previously [8] was adapted to yield firm spectral information for confirmation of the presence of EMP in these wines. Quantitative data for EMP in South African Sauvignon blanc wines are reported for the first time. This method utilises advantages inherent to LC-MS/MS to obtain very low detection limits.

7.2. Materials and methods

7.2.1. Chemicals and standards

High purity chemicals and solvents were used throughout. Methanol, ethyl acetate, MMP, EMP, IPMP, IBMP and SBMP were obtained from Sigma-Aldrich (Mulbarton, South Africa); formic acid, malic acid, phosphoric acid and tannic acid from Saarchem (Wadeville, South Africa); and acetonitrile, dichloromethane and hexane from Burdick & Jackson (Honeywell International, Muskegon, U.S.A.).

Standards were prepared by weighing an appropriate amount of reference material on an analytical balance. All dilutions were made using A-grade glassware. Standards were prepared in a solution of 40% acetonitrile. Wine samples for the recovery study were fortified volumetrically by adding appropriate amounts of working standards.

7.2.2. Samples

A total of 881 South African Sauvignon blanc wines, from all wine-producing regions and of vintages 1999-2011, were analysed. The vintage distribution of these samples was as follows (number of samples in parenthesis) 1999 (1), 2000 (1), 2001 (1), 2002 (12), 2003 (56), 2004 (199), 2005 (308), 2006 (58), 2007 (54), 2008 (109), 2009 (49), 2010 (15) and 2011 (18). Samples were obtained from submissions under the South African controlled appellations system (South African Wine and Spirit Board) as well as from export applications (Department of Agriculture, Forestry and Fisheries). General wine parameters such as pH, alcohol content, total acids and volatile acids were obtained from archived data that were used for certification of these products under the South African controlled appellations system. Malic acid concentrations were determined for a subset (n = 158) of samples using a liquid chromatography – ultraviolet-visible detection (LC-UV) method as outlined in Section 7.2.4.3.

7.2.3. Sample preparation

7.2.3.1. Extraction of MPs for quantitative analysis

The procedure used for analysis of MPs has been described previously [8]. Briefly, 500 mL wine was distilled utilising a 60 cm fractionating column and collecting the first 100 mL of distillate. The distillate was then extracted exhaustively with dichloromethane (10 mL, 5 mL and 5 mL, respectively). The combined dichloromethane fractions were acidified and concentrated by evaporation. Finally, the extract was reconstituted to 1 mL with a solution of 40% acetonitrile in water, homogenised, and transferred to a 1.8 mL vial for analysis.

7.2.3.2. Extraction of MMP and EMP for qualitative analysis

For spectral identification of MMP and EMP in Sauvignon blanc extracts, further semi-quantitative concentration and purification was performed using the following procedure. Approximately 0.9 mL of extract was removed from the autosampler vial and transferred to a 5 mL test tube. A 1 mL aliquot of hexane was added, followed by extraction on a vortex mixer at high speed for 1 minute. The hexane phase was then drawn off and transferred to a 5 mL test tube containing 1 mL of 10% formic acid in ethyl acetate. This solution was homogenised on a vortex mixer and evaporated under a gentle stream of nitrogen at room temperature to a volume of <100 µL. This residue was then redissolved in 200 µL of a solution of 50% acetonitrile in water. If required, acetonitrile was added drop-wise until a homogeneous solution was obtained following high-speed vortex mixing. Finally, the solution (~300 µL) was transferred to a total-recovery autosampler vial for analysis.

7.2.4. Chromatographic details

7.2.4.1. Quantitative analysis of MPs (HPLC program A)

Chromatographic analysis was performed as reported previously [8] using a Waters Alliance 2695 liquid chromatograph (LC) (Waters Corporation, Milford, U.S.A.) coupled to a Micromass Quattro Premier XE tandem quadrupole mass spectrometric detector (MS/MS) (Micromass Limited, Manchester, U.K.). Chromatographic separation was in reversed phase (RP) mode, employing a methanol and water gradient and a phenyl-hexyl column (Phenomenex Luna, Torrance, U.S.A.) with dimensions 250 x 4.6 mm (3 µm particle size), thermostatted at 40ºC. The gradient started at 35% methanol and increased to 80% in 18 minutes, followed by column clean-up (95% methanol for 2.4 minutes) and re-equilibration. The flow-rate was 1 mL/minute and the total run-time 25 minutes. This HPLC method is designated program A in the following discussions. A divert valve was used to direct the column effluent to the detector only between time 9.4 and 20.4 minutes, the rest being vented to waste. Variable injection volumes were used with the maximum injection volume limited to 100 µL. Ionisation was performed in positive ion mode with atmospheric pressure chemical ionisation (APCI). The corona current was 4.4 µA, cone voltage 34 V, and source and desolvation temperatures 150 and 200ºC, respectively. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, acquiring data for at least two ion transitions per analyte (given in units of *m/z*). Compounds were identified using retention times (t_R) as well as ion ratios as follows: MMP: t_R 10.6 min, MRMs 124.9 → 97.1, 56.2; EMP: t_R 13.8 min, MRMs 138.9 → 123.9, 83.0 and 111.0; IPMP: t_R 16.7 min, MRMs

152.9 → 137.9, 122.9; IBMP: t_R 18.3 min, MRMs 166.9 → 124.9, 123.9; and SBMP: t_R 18.7 min, MRMs 166.9 → 137.9, 122.9. Detailed mass spectral acquisition parameters and fragmentation energies are listed in Table 7.2.

Table 7.2. MRM transitions and peak identification criteria used for the LC-APCI-MS/MS determination of 3-alkyl-2-methoxypyrazines (ion ratios given in parenthesis).

Compound	Retention time (min)	Target ion (Da)	Product ion (Da)	Transition (ion ratio)	Collision energy (eV)
MMP	10.6	124.9	56.2	2° (2.4)	20
		124.9	97.0	1°	16
EMP	13.8	138.9	111.0	3° (3.4)	16
		138.9	83.0	2° (1.4)	16
		138.9	123.9	1°	20
IPMP	16.7	152.9	122.9	2° (1.1)	26
		152.9	137.9	1°	18
IBMP	18.3	166.9	123.9	2° (1.3)	22
		166.9	124.9	1°	16
SBMP	18.7	166.9	122.9	2° (1.9)	24
		166.9	137.9	1°	18

7.2.4.2. Qualitative analysis of EMP (HPLC program B)

For qualitative confirmation of EMP and MMP, sample extracts were further purified and concentrated as described above. The method for these analyses was identical to the one described above, except that the gradient separation was performed between 25 and 65% methanol, and an injection volume of 10 µL was used. Only EMP and MMP were then eluted in the mass spectral acquisition window to obtain critical separation of the target compounds from interfering peaks. In order to distinguish this procedure from the one described above that is used to separate five MPs, it will be designated HPLC program B in the following discussion.

7.2.4.3. LC-UV method for the determination of malic acid

Malic acid was determined by direct injection ion chromatography with UV detection. An Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) fitted with a quaternary pump, autosampler, column oven and UV-visible diode array detector was used. Chromatographic separation was performed in ion-exchange mode using a sulphonated (sulphonic acid form) styrene divinyl benzene-based column (Phenomenex, Torrance, U.S.A.) of dimensions 300 x 7.8 mm (8 µm particle size). The mobile phase was 0.05% (v/v) phosphoric acid in water. The column was thermostatted at 80°C and the flow-rate was 0.9 mL/minute. The run-time was 20

minutes and injection volumes of 0.1 µL were used while the chromatographic signal at 210 nm was recorded.

7.2.5. Data analysis and statistical methods

Factor Analysis (FA) and ANOVA were performed utilising Statistica (version 10, StatSoft, Inc., Tulsa, OK, USA) software. Pearson correlation coefficients were used to determine relationships between wine parameters and total methoxypyrazine concentration. Factor Analysis was used to further investigate correlation structures between variables and possible latent structures underlying the data. Parallel Analysis was used to determine the number of factors. Main effects ANOVA was used to test for differences in total methoxypyrazine concentrations between regions and districts taking vintage into account. Due to the non-normal nature of the data, log transformations were used.

7.3. Results and discussion

7.3.1. Performance and validation of the LC-APCI-MS/MS procedure

The liquid chromatography – atmospheric pressure chemical ionisation – tandem mass spectrometry (LC-APCI-MS/MS) method for the determination of methoxypyrazines in Sauvignon blanc wine used in this study is based on a procedure that was developed previously for this purpose [8]. The method combines the loading capacity inherent to LC with the sensitivity and selectivity of MS/MS in MRM mode as well as highly concentrated extracts to achieve very low LODs. The scope of the method reported previously was extended by the addition of MMP and EMP as target analytes (HPLC program A, Section 7.2.4.1).

The system response was linear (r^2 ~ 0.9999) for calibration standards over the range ~ 7 pg to 20 000 pg on-column, corresponding to a maximum sample concentration of 400 ng/L (for a concentration factor of 500 and 100 µL injection). The limits of detection (LOD) and quantification (LOQ) for the three major MPs (IPMP, IBMP and SBMP) were 0.03 ng/L and 0.10 ng/L, respectively. The LOD and LOQ for EMP were 0.10 ng/L and 0.33 ng/L, respectively. Although 15 pg (LOD of 0.33 ng/L) MMP could be detected in a standard, the recovery study showed that in real samples the signal was obscured by co-eluting matrix components at that concentration. Quantification of MMP was therefore only reliable above concentrations of 10 ng/L. Compound identification was achieved using retention times as well as the ion ratios for three MRM transitions (Table 7.2). For EMP three ion transitions were recorded, namely *m/z* 138.9 to 123.9, 111.0 and 83.0. For qualitative purposes, a tolerance of 2.5% in retention time

and 20% in the ion ratios (relative to calibration standards) was used [24]. Figure 7.1 show MRM chromatograms of four ion transitions used to quantify MPs in the extract of a Sauvignon blanc wine.

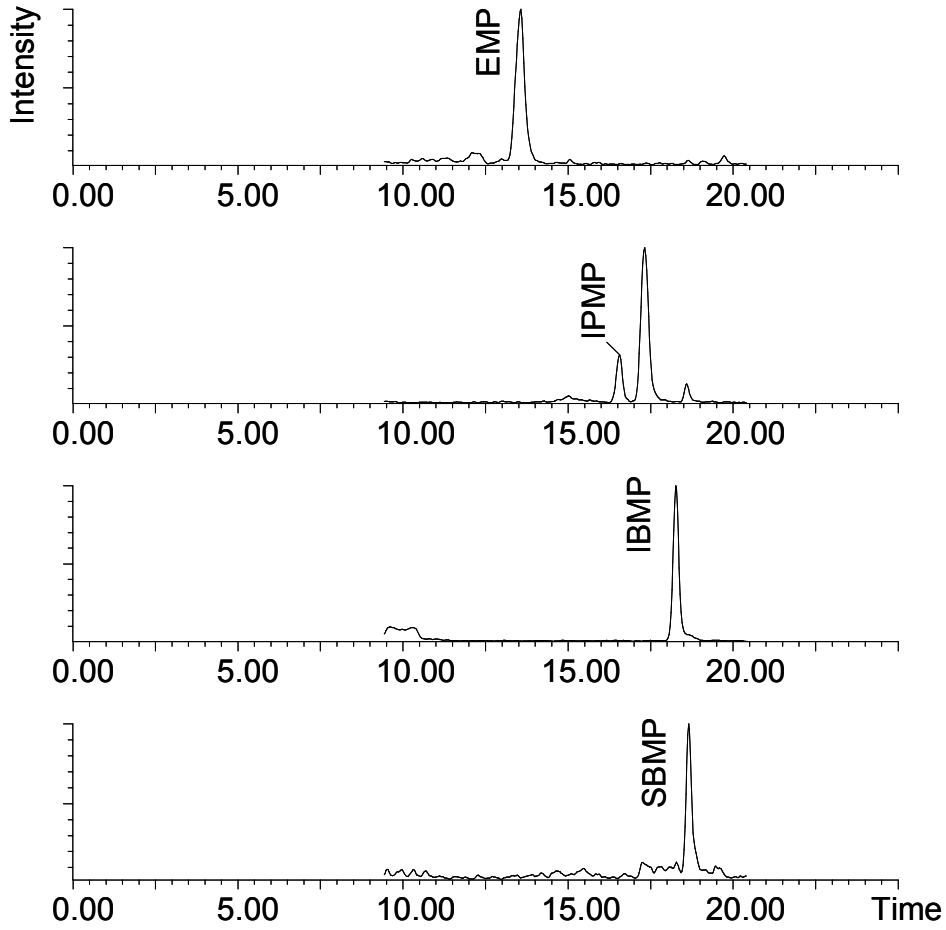


Figure 7.1. MRM chromatograms of four ion transitions used to quantify MPs in extracts of Sauvignon blanc wine: EMP (4.1 µg/L), IPMP (0.54 µg/L), IBMP (10 µg/L), SBMP (0.62 µg/L). The injection volume was 100 µL.

The accuracy of the method was assessed as the recovery of MPs from samples of Sauvignon blanc wine suitably fortified prior to the sample preparation step. Six replicate sets of recovery measurements were made at three levels of fortification (1, 10 and 100 ng/L). A full summary of the results of the recovery study, including overall recovery over the range of levels investigated, is presented in Table 7.3.

The reproducibility of the LC-APCI-MS/MS instrument, expressed as the relative standard deviation (RSD) of the peak area obtained for a standard (IBMP), was better than 2% (*n* = 8). The intra-assay precision, assessed as repeatability of the recovery, was better than 11% (expressed as RSD) at concentrations of 1 ng/L, 10 ng/L and 100 ng/L (IPMP, IBMP and SBMP only). The uncertainty of measurement associated with the procedure was estimated by calculating the range of the 95% confidence interval about the measurements performed in the recovery study (i.e. at levels of 1, 10 and 100 ng/L). Results are reported using the significant figure convention, according to which the result is reported by recording all the certain digits and the first uncertain digit [25]. As the limit of quantification of the procedure was determined to be a tenth of 1 ng/L, and the recoveries of the order 85 ± 5%, all results are reported in units of ng/L, using two significant figures.

The accuracy of the LC-APCI-MS/MS method was confirmed by comparison of quantitative results for the same samples with those obtained by an independent laboratory utilising a validated solid phase micro-extraction GC-MS method. Due to low sensitivity of the GC method (LOQ = 5 ng/L), only results for IBMP could be compared. Very good correlation was observed between these two sets of measurements (r^2 = 0.986, *n* = 6).

Table 7.3. Average recovery of MPs at three levels of fortification (1, 10 and 100 ng/L) as well as overall recovery (average recovery over all levels investigated). The relative standard deviation, as a percentage, is given in parenthesis.

Recovery (%)	MMP	EMP	IPMP	IBMP	SBMP
1 ng/L	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6
ave (% RSD)	-[a]	90.8 (28.2)	81.6 (10.3)	95.0 (11.3)	96.8 (5.0)
10 ng/L	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6
ave (% RSD)	65.7 (12.6)	79.6 (5.5)	80.0 (5.5)	87.3 (5.6)	80.4 (4.1)
100 ng/L	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6
ave (% RSD)	62.8 (6.9)	78.1 (4.2)	79.9 (5.1)	87.7 (3.2)	80.6 (3.4)
Overall	*n* = 12	*n* = 18	*n* = 18	*n* = 18	*n* = 18
ave (% RSD)	64.3 (10.2)	82.8 (18.6)	80.5 (7.0)	90.0 (8.4)	85.9 (10.1)

[a] MMP could not be accurately quantified at this concentration due to matrix interferences

7.3.2. Investigation of the occurrence of MMP and EMP in South African Sauvignon blanc wines

In order to investigate the occurrence of MMP and EMP, a subset of 10 South African Sauvignon blanc wines was used. EMP was detected and quantified in these samples using the MRM chromatogram of the primary ion transition (*m/z* 138.9 to 123.9) following analysis with HPLC program A. MMP was not detected (<10 ng/L) in any of the wines analysed. For EMP, the secondary and tertiary ion transition peaks (*m/z* 138.9 to 111.0 and 83.0) were obscured by co-eluting matrix elements so that firm spectral confirmation of the compound could not be obtained according to the criteria given in Table 7.2. However, since quantitative data pertaining to EMP in South African Sauvignon blanc wines are reported here for the first time, firm spectral compound identification was imperative. These extracts were therefore further purified as described in Section 7.2.3.2 and subsequently re-analysed with a modified HPLC program (program B, Section 7.2.4.2) to positively identify EMP in these wines. This procedure involved selective back-extraction of the MPs from the concentrated extracts, which served to further enrich the MPs while reducing the interfering sample matrix. The presence of EMP in each sample was inferred unambiguously from the correlation of retention time as well as two ion ratios for three ion transitions, compared with the reference standard. MRM chromatograms for three ion transitions obtained for the purified extract of a sample of Sauvignon blanc wine are displayed in Figure 7.2.

Augustyn *et al*. (1982) confirmed the presence of EMP in South African Sauvignon blanc wine [1], and Allen *et al*. (1998) tentatively reported EMP concentrations between 100 and 1 000 ng/L for two red wines [2]. The occurrence of MMP and EMP in wines was subsequently investigated by several further studies utilising headspace solid phase micro-extraction combined with GC and nitrogen-phosphorus selective detection [7,10] or tandem mass spectrometry [26] as well as liquid injection GC-MS [3], but method detection limits (ranging from 0.5 to 40 ng/L) did not allow reliable quantification of this compound. Therefore, apart from the single tentative and unconfirmed quantitative report [2], the current contribution represents the first report providing accurate quantitative data for this compound in wine. Spectral data is also presented to confirm the presence of EMP in these wines.

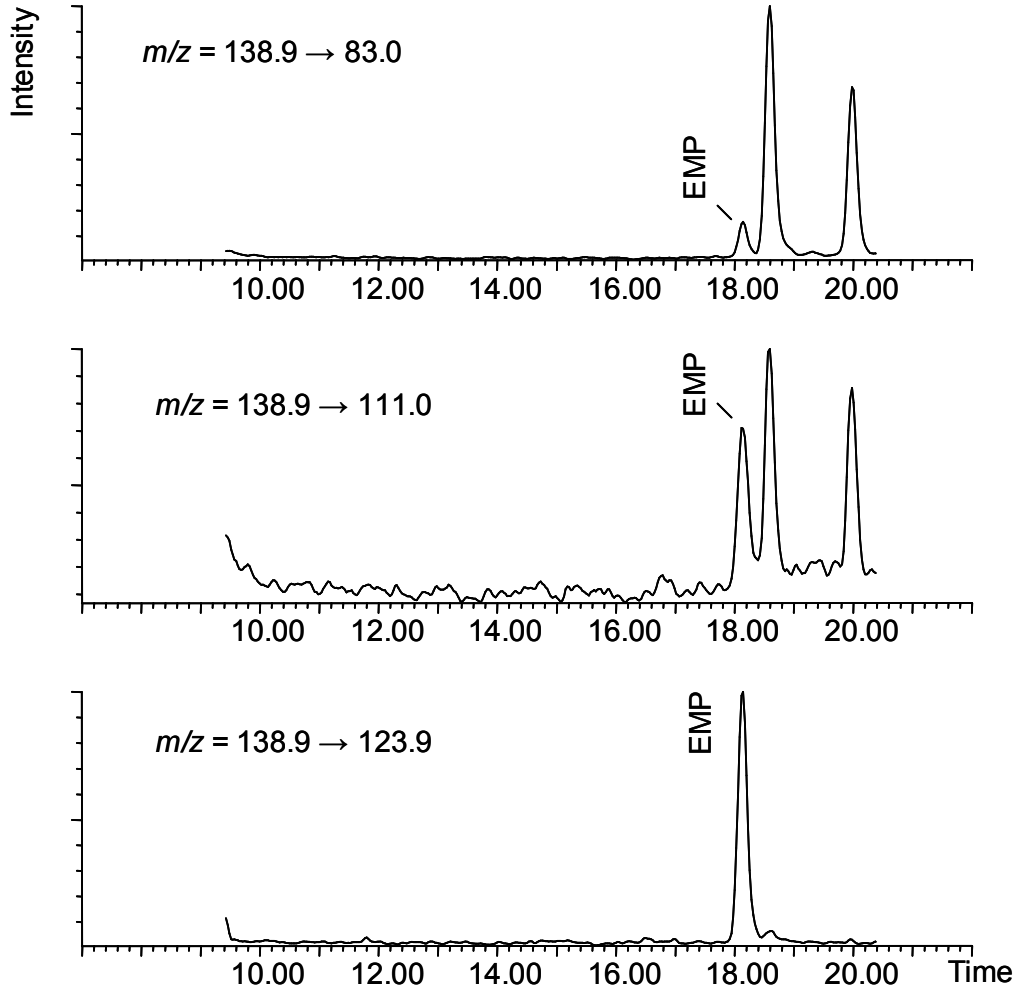


Figure 7.2. MRM chromatograms of three ion transitions obtained for EMP in purified extracts (sample preparation, described in Section 7.2.3.2) of Sauvignon blanc wine containing 4.9 ng/L EMP utilising LC-APCI-MS/MS (HPLC program B, see section 7.2.4.2).

The concentration of EMP in the 10 samples analysed varied between 1.1 and 4.9 ng/L, with an average of 2.9 ng/L (RSD of 48%, *n* = 10). At concentrations of this order of magnitude, EMP is not expected to affect the aroma of these wines considering that its odour threshold in water is 425 ng/L [2]. As is evident from Figure 7.3, the concentrations of EMP in these samples generally co-vary with those of the three other MPs (IPMP, IBMP and SBMP). This observation suggests that the synthesis and degradation of these four MPs may be affected by the same variables [19].

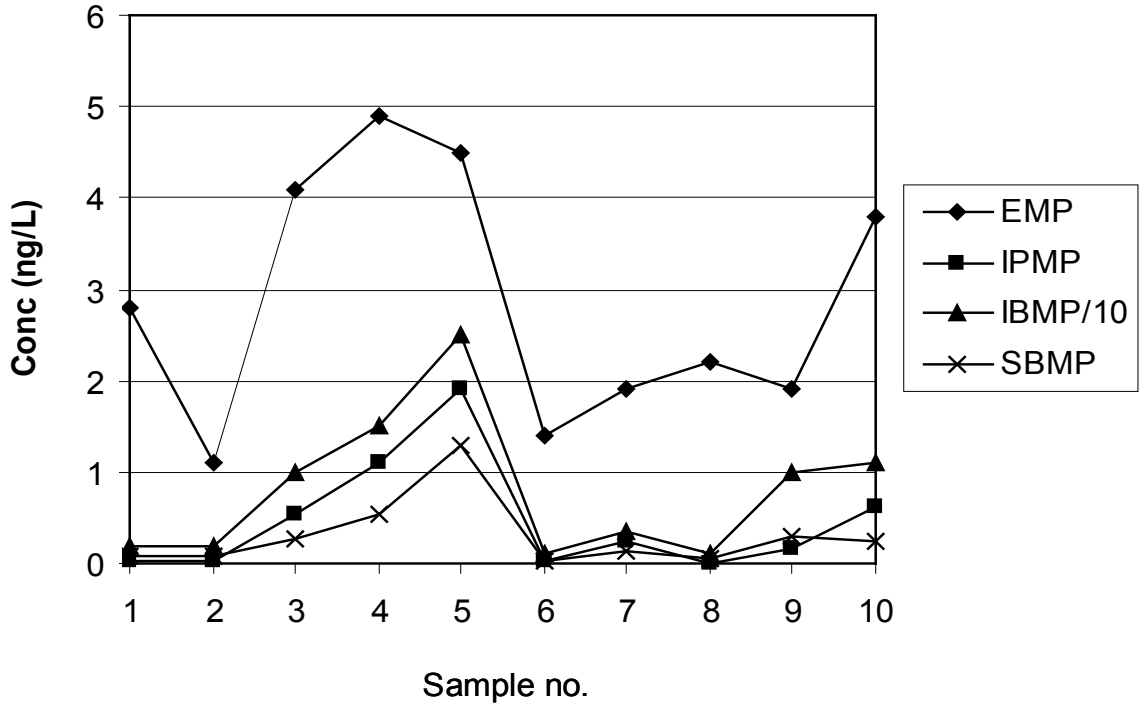


Figure 7.3. Concentrations of four MPs in 10 samples of South African Sauvignon blanc wine. IBMP attenuated (by a factor of 10) to enhance the readability of the figure.

7.3.3. Quantitative survey of the three principal 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wines

Three MPs (IPMP, IBMP and SBMP) have been found to contribute the characteristic vegetative aroma to Sauvignon blanc wine [1-7,9]. Although IBMP is invariably dominant, these compounds have similar odour detection thresholds, and their combined concentrations must be considered in the study of their flavour contribution to these wines [2]. At a combined concentration of 4-8 ng/L the herbaceous or vegetative aroma becomes evident in white wine, while the optimum concentration for Sauvignon blanc wine has been described as between 8 and 15 ng/L. Sensory perception at concentrations >30 ng/L are considered overpowering and out of balance [2].

In this study, data pertaining to the MP content of South African Sauvignon blanc wines accumulated over four years are reported, comprising a total of 881 samples from all wine producing regions of South Africa and covering vintages from 1999 to 2011. The IBMP

concentration in these samples ranged between 0.40 and 63 ng/L (RSD of 107.4%), with an average of 7.5 ng/L and median of 4.9 ng/L. Table 7.4 presents a concise summary of the IBMP concentrations of all wines, over these vintages, grouped according to geographical origin.

Table 7.4. IBMP levels (ng/L) in South African Sauvignon blanc wines from different geographical origins over vintages 1999 to 2011.

Region / district / ward	***n***	**Minimum**	**Maximum**	**Average**	**% RSD**
Breede River Valley	152	0.60	53	5.7	122.4
- Breedekloof [a]	20	0.89	26	8.0	81.6
- Robertson [a]	81	0.63	53	6.0	138.5
- Worcester [a]	42	0.60	17	4.7	83.2
Coastal Region	402	0.40	63	8.5	100.1
- Cape Point [a]	4	11	62	25	97.2
- Constantia [b]	25	6.1	29	12	46.1
- Darling [a]	18	4.6	39	14	53.7
- Paarl [a]	61	0.52	24	4.3	123.8
- Tygerberg [a]	33	0.74	38	11	74.6
- Stellenbosch [a]	162	0.76	63	7.8	113.9
- Swartland [a]	5	1.3	5.5	3.4	44.4
- Tulbagh [a]	3	1.9	2.2	2.0	7.5
Olifants River	6	3.5	45	17	104.6
- Lutzville Valley [a]	4	4.6	45	22	90.0
Cape Agulhas [a]	18	4.2	42	17	65.8
Overberg [a]	22	1.3	26	9.1	85.7
Walker bay [a]	18	1.2	25	7.2	85.4
Cederberg [b]	5	3.3	16	10	55.2
Prince Albert Valley [b]	3	0.81	4.2	2.0	97.8
Little Karoo	3	1.4	6.5	3.2	87.8
Western Cape [c]	250	0.48	44	6.1	112.0

[a] District (South African wine of origin scheme)

[b] Ward (South African wine of origin scheme)

[c] All wine producing areas of the Western Cape (South African wine of origin scheme)

The concentrations of IPMP and SBMP varied from below the LOD (0.03 ng/L) to 7.5 ng/L and 3.3 ng/L, respectively, and generally followed similar trends to those observed for IBMP. The ratios of IPMP to IBMP and SBMP to IBMP were approximately 10% in each case, and these displayed less variation than the overall IBMP concentration. These data follow similar trends compared to a previous study for a smaller set of wines [8]. However, the addition of 304 samples spanning a wider range of vintages and extending the representation of geographical

origins, improved the statistical representation of the South African Sauvignon blanc industry to increase the validity of conclusions drawn from the data. Furthermore, the malic acid content was determined for a subset of samples, which were used together with general wine parameters to investigate the correlation of these parameters with methoxypyrazine concentrations.

Two outstanding features in these data are the large variance (RSD of 108.5%) and asymmetric distribution (skewness of distribution around mean = 2.8) of the total MP concentration of these samples (where total MP concentration refers to the sum of IBMP, IPMP and SBMP). Based on the criteria reported by Allen *et al.* [2] the predicted flavour contribution of the total MPs to the analysed South African wines range from below recognition through to overpowering and out of balance. The majority of samples were at the lower end of the scale, with 38% of the wines classified as below recognition, while MP concentrations consistent with a recognisable vegetative aroma were found in 30% of the wines. In 18% of the wines the MP concentrations lie in the narrow concentration window where their contribution may be regarded as positively favourable to the vegetative Sauvignon blanc wine aroma. In 10% of the wines the concentrations were high and 4% contained MP concentrations that may result in an overpowering and unbalanced green pepper aroma [2]. It should also be noted that due to synergistic interactions between various aroma compounds, sensory properties and consumer preference of Sauvignon blanc wines are difficult to predict with great precision [27,28] and the flavour descriptions used in the current context refer to the probable effect that these compounds may have on the aroma of the wines.

7.3.3.1. Statistical investigation of the relationship between the viticultural parameters and 3-alkyl-2-methoxypyrazine content of South African Sauvignon blanc wines

In general, the timing of the harvest is a very important viticultural parameter as wine quality is limited by the properties of the fruit used in the vinification process. During the final stages of maturation, grape sugars and acids undergo important quantitative changes (see further) and the optimal harvest date ultimately depends on the intended style of the final product. At the same time, these parameters are also affected by climatic and viticultural factors [29]. Therefore, wine parameters such as acidity, pH and alcohol content may reveal information related to factors such as the maturity of the grapes at the time of harvesting as well as climatic and viticultural conditions. Since wine MP concentrations are also strongly and systematically influenced by these viniviticultural factors, their correlation with wine parameters such as total

acidity, volatile acidity, malic acid, pH, and alcohol content were investigated for a subset (*n* = 158) of wines using two-dimensional and multivariate data analysis techniques. In addition, differences between vintages and regions of origin were elucidated (for the entire dataset, *n* = 881) using methoxypyrazine data for the three principal congeners in order to assess the effects of annual climatological variations and geographic differences.

Apart from the change in sugar content, the reduction of acidity is quantitatively the largest chemical change to occur in grapes during ripening. Tartaric and malic acids are quantitatively the most abundant grape acids, accounting for approximately 70-90% of the berry acid content. The concentrations of these acids are determined by complex factors, but certain trends are generally observed. While the tartaric acid content remains relatively stable after *véraison*, the malic acid content of grapes declines drastically during maturation. The rate of decline in berry malic acid is cultivar dependent and is also strongly affected by environmental factors. It is generally accepted that grapes grown in hot climates often metabolise most of their malic acid before harvest, whereas grapes grown in cooler climates retain most of their malic acid into maturity [29]. As a result, a relatively high wine malic acid concentration may indicate that the grapes were harvested earlier or were grown in cool conditions, both of which are also factors that are associated with higher levels of wine methoxypyrazines [4,6,14,17-19]. This hypothesis is supported by a study which reported that the breakdown of malic acid and IBMP in grapes during ripening are highly correlated (r^2 of 0.90 to 0.99), irrespective of soil type, grape variety or vintage [6]. The malic acid content of a wine may therefore reveal information regarding cultivation micro-climate and maturity of the grapes and by extension, the methoxypyrazine potential of the product.

In addition, it has also been reported that excessive vigour (the relationship between foliage and fruit) is consistently correlated with reduced acidity and high wine pH [29]. Vigour, in turn, is also correlated with higher levels of wine methoxypyrazines, mainly as a result of shading of bunches during ripening [14]. Low wine total acidity and high pH, when resulting from increased vigour, may therefore be correlated with higher levels of wine methoxypyrazines. These phenomena (early harvesting and vigour) are generally associated with higher concentrations of wine methoxypyrazines, but they have opposing effects on wine pH and acidity. However, since the level of tartaric acid is relatively stable after *véraison*, the ratio of tartaric to malic acid may be more significant in predicting the concentrations of wine MPs.

These hypotheses were investigated to determine whether the methoxypyrazine content of Sauvignon blanc wines were correlated with wine parameters such as malic acid concentration, acidity, pH and alcohol content. Note that reducing sugars are not expected to provide useful information in these analyses since the practice of sweetening may alter this parameter independent of production conditions.

Two-dimensional plots of wine parameters such as alcohol content, pH, total acids, volatile acids and malic acid *vs.* the total wine MP concentration for the subset of 158 samples produced considerable scattering of data-points. Alcohol content (Pearson correlation coefficient, r = 0.04, p 0.65) and pH (r = 0.05, p 0.52) did not show significant co-variance with total MP content, whereas statistically significant positive correlations were observed between the variables total acids (r = 0.29, p <0.01), volatile acids (r = 0.30, p <0.01) and malic acid (r = 0.34, p <0.01) and total wine MP concentration. Samples with high levels of total acids, volatile acids and malic acid therefore generally also contained higher concentrations of MPs, while alcohol content and pH displayed little systematic variance as a function of MP concentration. Despite these observations, the low correlation coefficients indicate significant scatter in the data, implying that additional factors also affect these variables.

Nature is multivariate in the sense that particular phenomena, such as wine methoxypyrazine content, usually depend on several factors, such as time of harvest, vigour, solar radiation, canopy microclimate, mesoclimate, as well as oenological and viticultural practices. Each of these production factors also affects the general wine parameters such as alcohol content (Alc), pH, TA, VA and malic acid. The multivariate data analysis technique Factor Analysis was therefore used to determine whether intrinsic correlations exist between wine MP concentrations and these parameters, since this method it is suitable for reducing the dimensionality of data by extracting interpretable factors. By reducing multivariate data to a few sets of factors of physical significance, the visual representation of these relationships is enhanced and meaningful interpretation of the data facilitated. In this way the underlying correlations and qualitative features in the data can be identified.

In the first instance, Parallel Analysis was performed on the data subset (n = 158) for which malic acid was determined to give an indication of the number of factors underlying the original data. Two factors were identified that explained 60.5% of the total variance. The loadings plot for these two factors is displayed in Figure 7.4. It is evident from this analysis that the

concentrations of the three principal wine MPs are highly correlated, and mainly responsible for the variation described by Factor 1 (F1). This implies that for wines rich in IBMP, the concentrations of IPMP and SBMP are invariably correspondingly high, and *vice versa*. This information suggests that their occurrence in wine is regulated by the same viniviticultural variables.

Alcohol content and pH displayed a negative correlation. These variables showed little co-variance as a function of MP concentration (F1), but are rather associated with the variance described by Factor 2 (F2). TA and VA, practically to the same extent, are also positively correlated with MP concentration and therefore with the variance described by F1. Of all the wine parameters, TA shows the highest correlation with wine MPs – a phenomenon that may be explained by noting that relative early harvesting results in higher grape acidity and is also favourable for producing wines containing high levels of MPs. On the other hand, relatively late harvesting and warm growing conditions may produce lower grape acidity and also lower wine MPs. The correlation of malic acid with wine MPs supports this observation, as malic acid retention may be ascribed to early harvesting or cool growing conditions – factors that are conducive to high wine MPs. The correlation of malic acid levels with MPs observed here is, however, not as clearly defined as the association reported by Roujou De Boubee *et al.* [6].

Acetic acid is the main volatile acid in wine, and in the absence of spoilage, it occurs principally as a by-product of yeast and bacterial metabolism [29]. It has been reported that different yeast strains have an effect on the yield of wine MPs from the juice [4,21]. The correlation of VA with MP levels may therefore possibly be ascribed to the effect of different yeast strains on the production of acetic acid and MPs in the wine. This assumption is also supported by the correlation of VA and alcohol content, since it is well known that different yeast strains cause differences in the production wine ethanol.

The positive correlation of malic acid content with pH indicates cool growing conditions, conducive to the retention of grape malic acid into maturity, while simultaneously leading to low total acids and high wine pH. The same phenomenon also explains the negative correlation of malic acid with alcohol content, as cool growing conditions, which favour malic acid retention, also produce lower concentrations of sugars and less alcoholic wines. The fact that pH is not correlated at all with F1 might be due to the opposing effects of vigour (lower acidity) and early harvesting (higher acidity) on wine pH, while both these factors are conducive to higher wine

MPs. The negative correlation of pH and alcohol content confirms the validity of these assumptions, as cool growing conditions and vigour lead to the production of lower acids (higher wine pH) and lower sugars (lower wine alcohol) at the same time. On the other hand, riper grapes would contain higher concentrations of sugars and lower concentrations of acids, yielding higher pH and more alcoholic wines. The fact that this phenomenon (wines with high pH and high alcohol content) is not observed in this analysis demonstrates the involvement of cool growing conditions and vigour in the production of these wines.

Surprisingly, a positive correlation between alcohol content and total acids was found for the analysed wines. It would be expected that higher acidity, resulting from early harvesting and cool growing conditions, would at the same time also lead to the production of lower sugars and less alcoholic wines.

From these data it would appear that climactic conditions, grape ripeness and vigour all play a determinative role in the MP content of Sauvignon blanc wines. Furthermore, while general wine parameters such as TA, VA and malic acid concentration show some correlation with MP levels, they are not absolute indicators for MP content of Sauvignon blanc wines due to the complex interplay between variables affecting MP concentration in the final wine.

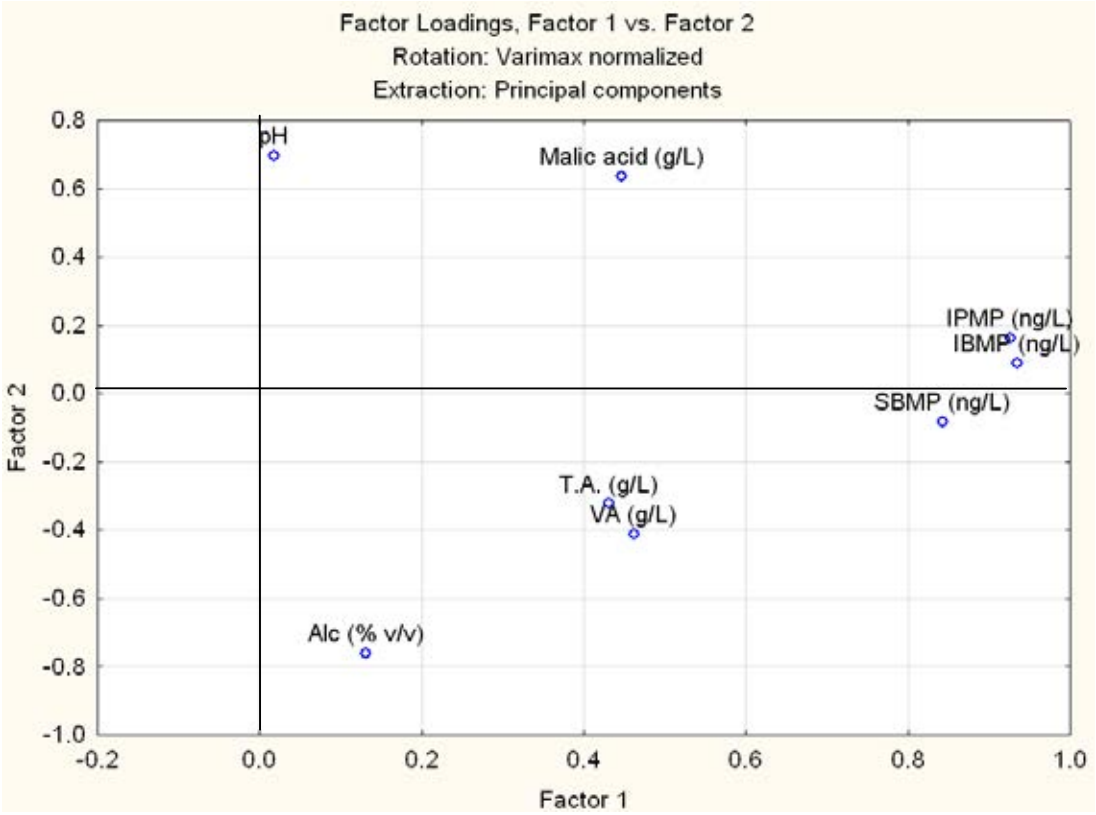


Figure 7.4. Loadings plot for Factor Analysis of two principal factors obtained for the data subset of 185 wines.

7.3.3.2. Variations in 3-alkyl-2-methoxypyrazine content of South African Sauvignon blanc wines as a function of geographical origin

In wine production, certain factors that have an effect on the product are related to its geographical origin. Aspects such as soil influences (for example geological origin, texture, structure, drainage and water availability, depth, nutrient content and pH, organic content and colour), topographic influences (for example solar exposure, wind direction, frost and winter protection, altitude and drainage) and atmospheric influences (such as temperature, solar radiation, wind and water availability) impact on the final product to varying extents. This point is amply illustrated by noting that regions affected by continental influences may have more than twice the average day-night temperature variation compared to an equivalent maritime region. In addition, seasonal climatic variations may manifest changes in the product as a function of vintage [29]. In grapes, the concentrations of MPs are strongly affected by *terroir* (soil, climatic

and viniviticultural influences) [2,3,5,6,14,16,17,18,20]. The systematic effects of geographical origin and vintage on the total MP concentration of South African Sauvignon blanc wines were therefore investigated using main effects ANOVA.

Firstly, classification according to region of origin was investigated. In the South African wine of origin scheme, wines may be certified for region of origin by classification into regions, districts or wards (see also Table 7.2). Of these categories, region is the broadest category of classification. Samples from all vintages in the dataset were therefore grouped according to region where appropriate, while all non-specific origins (such as Western Cape) and minor regions (for which insufficient data were available) were removed. A data subset of 554 samples was obtained, of which 145 samples originated from Breede River Valley and 385 samples from Coastal Region. This subset was analysed by ANOVA to determine whether these origins can be distinguished based on the total MP concentration of these wines. The mean of the Breede River Valley group was lower than that of Coastal Region (p <0.01). This result possibly indicates slightly warmer growing conditions prevalent in the Breede River Valley region compared to the Coastal Region. Despite the relatively wide limits for the 95% confidence interval, which indicate significant variation in MP levels, statistically significant differences in mean values between these two regions were found.

ANOVA was subsequently used to elucidate grouping of samples from various different districts located in the greater Breede River Valley and Coastal Region, using total MP data. By performing main effects ANOVA using wine samples for those districts that are represented by a statistically significant number of samples and over vintages 2003-2011, significant differences between these districts were elucidated, and in this way the effect of variation over vintages was taken into account. Figure 7.5A illustrates that there are clear differences between some districts in terms of total wine MP content. For example, Overberg and Tygerberg were clearly distinct from Paarl and Robertson, based on higher levels of MPs. Overberg and Tygerberg are two districts located in relative close proximity to the Atlantic Ocean and this result possibly indicates favourable viniviticultural conditions for Sauvignon blanc production at these locations. On the other hand, Paarl and Robertson represent districts that are characterised by warmer climates and this analysis indicates that they generally produce wines that contain lower levels of MPs. Since this analysis represents a large number of samples (n = 382) and is independent of vintage, it is clear that statistically significant differences (p <0.01) exist in wine MP concentrations between different districts. This indicates that certain districts inherently lend

themselves to the production of Sauvignon blanc wines of higher MP content. This observation is in agreement with the work of Marais *et al*. who also observed the suitability of distinct localities for Sauvignon blanc production [14].

Note that discrepancies in the number of samples between the districts, as well as variance in the data for each district, affect the results reported in Figure 7.5A. For example, Stellenbosch represents the largest number of samples (*n* = 158), while Overberg (*n* = 20) is represented by much lower numbers, which explains the larger 95% confidence interval for the latter. As before, these significant differences can not be used to absolutely classify wines according to districts of origin due to the relatively large variation in each group.

7.3.3.3. Variation in 3-alkyl-2-methoxypyrazine content of South African Sauvignon blanc wines as a function of vintage

The effect of climatic variations on total wine MP concentrations was evaluated by comparing mean MP levels in the total dataset between vintages. Samples from 2003 to 2009 (*n* = 382) were compared, controlling for district in a main effects ANOVA. From the information in Figure 7.5B, it is clear that in 2007 and 2009 wines with relatively high concentrations of MPs were produced. Since the data are independent of geographical origin, this result suggests that favourable climatological conditions (such as lower temperatures, higher rainfall and/or lower solar radiation) were prevalent in the 2007 and 2009 seasons compared to other vintages.

Finally, it should be noted that the commercial products analysed in this study, although labelled as single varietal wines, may contain between 15 and 25% of other varieties, (depending on vintage). In addition, wines certified for vintage may contain up to 15% of wine from the preceding or subsequent years [30]. This additional variability can certainly contribute to the observed variance in MP concentrations, particularly considering that the grapevine synthesis of MPs is highly dependent on genotype [31] and because of climatic variations between vintages [3,15].

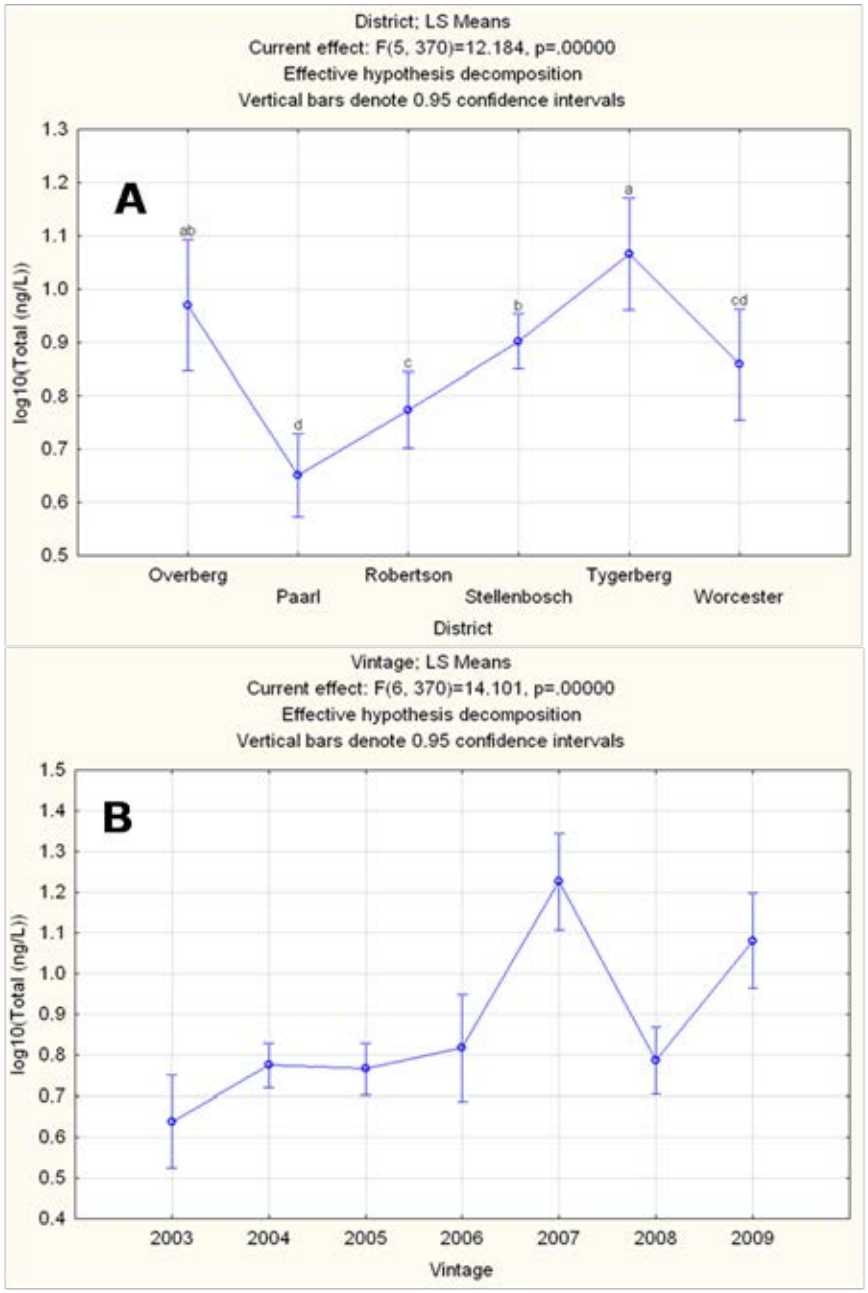


Figure 7.5. ANOVA results for determining significant differences between (A) districts, and (B) vintages between 2003 and 2009, based on total wine MP concentration.

7.4. Conclusions

In this study an LC-APCI-MS/MS method was applied to generate the first extensive database on the concentrations of 3-alkyl-2-methoxypyrazines in 881 samples of South African Sauvignon blanc wines spanning 13 vintages and all the major wine producing regions of the country. The total MP concentration (sum of IPMP, IBMP and SBMP) in these samples ranged from 0.52 to 72 ng/L, suggesting that the typical green pepper aroma of these products may range from below recognition through to overpowering and out of balance. Their combined concentrations exceeded the recognition threshold for the vegetative aroma in more than 60% of the samples analysed, demonstrating the extent of involvement of these compounds in the typical aroma of the cultivar.

The first quantitative data for 3-ethyl-2-methoxypyrazine in Sauvignon blanc wines is reported, and the presence of the compound was confirmed in a subset of 10 samples using modified extraction and analysis procedures. The concentrations of 3-ethyl-2-methoxypyrazine were two orders of magnitude below its odour detection threshold, which makes it highly unlikely that this MP contributes significantly to the aroma of these wines.

Statistical analysis of the total MP content data using Factor Analysis show that the concentrations of IPMP, IBMP and SBMP are highly correlated, suggesting that their occurrence in Sauvignon blanc wines are regulated by the same factors. In addition, Factor Analysis revealed that methoxypyrazines are correlated with total acidity, volatile acids and malic acid. Higher acidity may result from earlier harvesting, while higher levels of malic acid in particular indicate cooler climates or early harvesting, viniviticultural conditions that are also favourable for the production of wines containing higher levels of MPs. The observed correlation between MP levels and volatile acids may possibly be ascribed to the effect of different yeast strains on the yield of MPs from the juice.

Statistical analysis using ANOVA showed significant differences between wines from different geographical origins (independent of vintage) and vintages (independent of geographical origin). Especially the districts Overberg and Tygerberg appear to be particularly suited for the production of varietal-typical vegetative Sauvignon blanc wines, as they displayed higher average total MP concentrations, while across all regions the 2007 and 2009 vintages also produced wines that contained higher average levels of MPs. The data presented in this paper shed much-needed light on the MP content of representative South African Sauvignon blanc wines and the factors affecting the levels of these important aroma compounds.

References

[1] O.P.H. Augustyn, A. Rapp, C.J. van Wyk, S. Afr. J. Enol. Vitic. 3 (1982) 53. .

[2] M.S. Allen, M.J. Lacey, Chemistry of wine flavor, Oxford University Press, New York, U.S.A. (1998), Chapter 3, p 31.

[3] M.J. Lacey, M.S. Allen, R.L.N. Harris, W.V. Brown, Am. J. Enol. Vitic. 42 (1991) 103.

[4] J. Marais, Wynboer, 12 (2004) 69.

[5] J. Marais, P. Minnaar, F. October, Wynboer, 2 (2004).

[6] D. Roujou De Boubee, C. Van Leeuwen, D. Dubourdieu, J. Agric. Food Chem. 48 (2000) 4830.

[7] C. Sala, M. Mestres, M.P. Marti, O. Busto, J. Guasch, J. Chromatogr. A 953 (2002) 1.

[8] P. Alberts, M.A. Stander, A. de Villiers, J. Agric. Food Chem. 57 (2009) 9347.

[9] M.S. Allen, M.J. Lacey, R.L.N. Harris, W.V. Brown, Am. J. Enol. Vitic. 42 (1991) 109.

[10] C. Sala, M. Mestres, M.P. Marti, O. Busto, J. Guasch, J.Chromatogr. A 880 (2000) 93.

[11] H-G. Schmarr, S. Ganß, S. Koschinski, U. Fischer, C. Riehle, J. Kinnart, T. Potouridis, M. Kutyrev, J. Chromatogr. A 1217 (2010) 6769.

[12] J.A. Maga, C.E. Sizer, J. Agric. Food Chem. 21 (1973) 22.

[13] K.E. Murray, F.B. Whitfield, J. Sci. Food Agric. 26 (1975) 973.

[14] J. Marais, J.J. Hunter, P.D. Haasbroek, S. Afr. J. Enol. Vitic. 20 (1999) 19.

[15] L.D. Falcăo, G. De Revel, M.C. Perello, A. Moutsiou, M.C. Zanus, M.T. Bordignon-Luiz, J. Agric. Food Chem. 55 (2007) 3605.

[16] C. Sala, O. Busto, J. Guasch, F. Zamora, J. Sci. Food Agric. 85 (2005) 1131.

[17] K. Hashizume, T. Samuta, Am. J. Enol. Vitic. 50 (1999) 194.

[18] C. Sala, O. Busto, J. Guasch, F. Zamora, J. Agric. Food Chem. 52 (2004) 3492.

[19] I. Ryona, S. Leclerc, G.L. Sacks, J. Agric. Food Chem. 58 (2010) 9723.

[20] M. Maggu, R. Winz, P.A. Kilmartin, M.C.T. Trought, L. Nicolau, J. Agric. Food Chem. 55 (2007) 10281.

[21] G.J. Pickering, M. Spink, Y. Kotseridis, D. Inglis, I.D. Brindle, M. Sears, A-L. Beh, Australian Journal of Grape and Wine Research 14 (2008) 230.

[22] T.G. Galvan, S. Kells, W.D. Hutchison, J. Agric. Food Chem. 56 (2008) 1065.

[23] A. Blake, Y. Kotseridis, I.D. Brindle, D. Inglis, M. Sears, G.J. Pickering, J. Agric. Food Chem. 57 (2009) 4680.

[24] M.J. O'Keeffe, S. Martin, L. Regan, Anal. Chim. Acta 483 (2003) 341.

[25] D.A. Skoog, D.M. West, F.J. Holler, Fundamentals of Analytical Chemistry, Saunders College Publishing, Orlando, U.S.A. (1996). p 660.

[26] R. Godelman, S. Limmert, T. Kuballa, Eur. Food Res. Technol. 227 (2008) 449.

[27] A. Escudero, E. Campo, L. Farina, J. Cacho, V. Ferreira, J. Agric. Food Chem. 55 (2007) 4501.

[28] E.S. King, P. Osidacz, C. Curtin, S.E.P. Bastian, I.L. Francis, Australian Journal of Grape and Wine Research 17 (2011) 169.

[29] R.S. Jackson, Wine science: Principles, practice and perception, Academic Press, San Diego, U.S.A. (2000).

[30] Liquor Products Act, Government Gazette, South Africa, Act 60 (1989).

[31] A. Koch, C.L. Doyle, M.A. Matthews, L.E. Williams, S.E. Ebeler, Phytochemistry 71 (2010) 2190.

Chapter 8

Advanced ultra high pressure liquid chromatography – tandem mass spectrometric methods for the screening of red wine anthocyanins and derived pigments *

* Published as P. Alberts, M.A. Stander, A. de Villiers, J. Chromatogr. A 1235 (2012) 92-102.

8.1. Introduction

The colour of red grapes and wine is attributed largely to the anthocyanins (anthocyanidin-glycosides). Apart from their important role in the aesthetic perception and quality of red wines, anthocyanins may also serve as markers for wine authentication and possess beneficial biological properties, which have led to increasing recent interest in their analytical determination [1,2].

Anthocyanins belong to the flavonoid family and exist as glycosides through conjugation of anthocyanidins with primarily glucose. These compounds exhibit a large structural diversity due to the substitution pattern of the anthocyanidin base as well as variations in the position, number and acylation of sugar moieties. In *Vitis vinifera* varieties pigments are mainly in the 3-O-glucoside form, whereas in other *Vitis* species 3,5-diglucosides also occur. Five anthocyanidin bases are present in red grapes, namely, malvidin, delphinidin, peonidin, cyanidin and petunidin. Malvidin-3-O-glucoside is the predominant pigment in *Vitis vinifera* varieties [2-4]. During maturation and ageing of red wines, anthocyanins undergo addition reactions with other wine constituents to yield pigments that impart important changes to the product, notably in terms of colour and taste. They may react with flavanols, either directly or mediated by other compounds such as acetaldehyde [5-8]. Wine constituents possessing a polarisable double bond (such as pyruvic acid [9,10], acetaldehyde [11] or vinylphenol [12]) also undergo cyclo-addition reactions with anthocyanins to add a pyran ring to the anthocyanidin base [5,7,9,10]. The pyranoanthocyanins possess a tawny colour, and are relatively stable to temperature and pH effects as well as to sulphur dioxide bleaching [3,13,14]. Pyranoanthocyanins may further react with other wine constituents such as vinyl-flavanols to produce blue pigments named portisins [15]. All of these anthocyanin-derived pigments contribute to the observed change in wine colour from the initial purple-red to a brick-red hue characteristic of aged wines, and there is a concurrent decrease in astringency due to the involvement of tannins in these processes [13].

Reversed phase liquid chromatography (RP-LC) is the most widely used separation technique for the study of anthocyanins. These compounds are subject to pH dependent inter-conversions between various molecular forms and a highly acidic mobile phase (pH <2) is therefore required to ensure that they are maintained predominantly in the flavylium cationic form for maximum chromatographic efficiency [2]. However, even at low pH some inter-conversion between the anthocyanin flavylium cationic and carbinol pseudo-basic forms occurs [16]. The slow rate of this inter-conversion reaction dictates relatively low optimal mobile phase velocities (~0.1 mm/second) for their separation. Chromatographic efficiency may further be improved by using small-particle columns (~1.7 µm) and elevated

temperatures (~50°C) to increase the rate of inter-conversion reactions as well as mass transfer [16]. De Villiers *et al.* [16] demonstrated that thermal degradation of anthocyanins is avoided at this temperature, while gradient separation on 1.7 µm phases at elevated temperature provides highly efficient separation of wine anthocyanins [17].

Diode array detection (DAD) and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) are the most widely used chromatographic detection techniques for the study of anthocyanins. LC-DAD is inherently suited to provide information on the colour of these compounds and can also tentatively distinguish between the main phenolic structures, since these displays unique ultraviolet-visible (UV/Vis) absorption spectra, characterised by absorption in the visible range around 500 nm. MS provides benefits such as increased sensitivity and structural information compared to LC-DAD. Various MS instruments have been applied to anthocyanin analysis, varying between simple quadrupole and more advanced tandem and high-resolution instruments such as triple quadrupole and quadrupole-time-of-flight (QTOF) systems. MS/MS is particularly suited for structure elucidation and compound identification since information pertaining to the aglycone moiety, type and number of sugars and other substituents can be obtained [1]. Since few reference standards for anthocyanins are available (in particular, no standards are available for derived wine anthocyanins), compound identification is often tentative and is usually based on RP-LC elution order, UV/Vis spectra as well as mass spectral information (molecular weight, accurate mass and/or MS/MS fragmentation [1]). However, due to the large numbers and structural similarity of these compounds, complete chromatographic separation of wine anthocyanins is not attainable. Moreover, compound identification is complicated by the fact that some non-anthocyanin phenolic compounds have similar mass spectral characteristics.

Direct infusion MS has also been described for the study of these compounds [14,18]. These techniques uses direct introduction of purified sample extracts, producing a rapid and sensitive methodology for qualitative and semi-quantitative determination of anthocyanins. However, a number of anthocyanins and derived products have identical mass spectral properties, which render them indistinguishable by MS alone. In contrast, chromatographic separation in combination with MS provides adequate resolution to identify these compounds with higher accuracy and may also reduce possible ionisation suppression effects known to be associated with electrospray ionisation. However, since complete chromatographic resolution is not ensured, it is imperative that mass spectral information, in addition to the molecular ion and mass of the sugar moiety, is available for unambiguous compound identification.

In this study, a methodology is proposed for the analysis and unambiguous identification of the principal anthocyanins and derived compounds in red wines. An ultra high pressure liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) method utilising optimised chromatographic parameters [16,17] was developed to investigate these compounds in *Vitis vinifera* cv. Pinotage wines. The anthocyanins and anthocyanin-derived compounds are detected with great selectivity using MS/MS in neutral loss scanning mode. By performing concurrent product ion scans, the aglycone cation moieties of each detected compound can be identified unambiguously *via* their characteristic fragmentation patterns under high collision energy conditions [14]. For each detected compound, identification is therefore based on the well-known RP-LC elution order, molecular weight of the compound and mass of the sugar moiety (obtained in the neutral loss experiment), as well as identification of the aglycone cation (*via* spectral information yielded by the survey scan experiment). Three families of anthocyanin-derived pigments were investigated in this study, namely anthocyanins, pyranoanthocyanins and flavanol-anthocyanin condensation products.

8.2. Experimental

8.2.1. Materials

Malvidin-3,5-diglucoside was obtained from Sigma-Aldrich (Mulbarton, South Africa), malvidin-3-glucoside chloride (MVG, Oenin chloride) from Extrasynthese (Genay, France) and cyanidin-3-O-glucoside from Polyphenols Laboratories (Sandnes, Norway). Standards were prepared by weighing reference material on an analytical balance and all dilutions were made using volumetric glassware in a solution of 10% ethanol in water. All other chemicals and solvents were HPLC grade, and were supplied by Sigma-Aldrich (Mulbarton, South Africa).

8.2.2. Samples

South African wines were commercial products of the cultivar *Vitis vinifera* cv. Pinotage, aged between 1 and 4 years. Two German Dornfelder hybrid-cultivar wines were also studied since these contain higher levels of diglucoside anthocyanins. The Dornfelder wines were obtained from import submissions (South African Department of Agriculture, Forestry and Fisheries). Samples were filtered using 0.22 µm HVLP syringe filters (Millipore, Milford, MA, USA) prior to analysis.

8.2.3. UHPLC-MS/MS analysis

A Waters Acquity UHPLC system (Waters Corporation, Milford, MA, U.S.A.) incorporating a binary pump, vacuum degasser, autosampler, column oven and Micromass Xevo tandem quadrupole mass spectrometric detector (Manchester, U.K.) equipped with ESI probe was

used. Reversed phase separation was performed using an Acquity BEH C18 column (2.1 x 100 mm, 1.7 µm particle size) at a temperature of 50°C. Mobile phase A was 7.5% formic acid in water and mobile phase B acetonitrile. The gradient started with 1% B isocratically for 0.5 minutes followed by a linear increase to 15% at 15 minutes, 23% at 20 minutes and 28% at 25 minutes. Column clean-up at 100% B then followed for 1 minute followed by re-equilibration for 4 minutes (total run-time of 30 minutes). The flow-rate was 0.1 mL/minute throughout and injection volumes of 10 µL were used. The source conditions were as follows: capillary voltage 2.5 kV and cone voltage 15 V. The desolvation temperature was 500°C and the desolvation and cone gas (both nitrogen) flows were 1000 and 50 L/hour, respectively. Two different MS data acquisition modes were used. Firstly, the instrument was operated in neutral loss mode, monitoring four neutral loss functions: *m/z* 162, 204, 308 and 324. The mass range for the first three neutral losses was 400-900 *m/z*, and *m/z* 430-1200 for the 324 neutral loss scan. The collision energy was 25 V for the neutral loss experiments. Secondly, four methods were used to obtain product ion spectra for the peaks detected in the neutral loss experiments for qualitative information. For this purpose, four separate survey scan methods were used for each of the four neutral losses (*m/z* 162, 204, 308 and 324), and the instrument was set at a low trigger sensitivity in each case. In this mode, the instrument switches from neutral loss mode to product ion scan mode using a scan range of *m/z* 100-1500 at a collision energy of 50 V. The limit of detection (LOD) of the neutral loss method was ~0.1 mg/L, based on a signal-to-noise ratio of 3:1 determined for malvidin-3-O-glucoside. The survey scan method yielded consistent qualitative information of the aglycone cations at concentrations above 5 mg/L, as determined for malvidin-3-O-glucoside.

8.2.4. High resolution MS/MS analysis

A Waters Synapt G2 QTOF mass spectrometer was used for high resolution LC-MS/MS analysis to confirm the molecular formulas of fragment ions. The LC method and source conditions were identical to those described above for UHPLC-MS/MS analysis except that a collision energy of 60 V was used. Leucine encaphalin (*m/z* = 556.2771) was used as reference (lock) mass and the instrument was calibrated with sodium formate. The resolution of the instrument is 20 000-24 000 in the mass range that data were acquired in and the accuracy was better than 2 ppm.

8.3. Results and discussion

Direct infusion MS provides a rapid and sensitive methodology for qualitative and semi-quantitative determination of anthocyanins. However, a number of wine anthocyanins have identical mass spectral properties and can therefore not be distinguished utilising MS exclusively. For example, the isomers of the 3-O-coumaroylglucosides and 3-O-

caffeoylglucosides cannot be differentiated by MS. The 3-O-caffeoylglucosides have identical molecular ion masses, aglycone cations and sugar moiety masses compared to the corresponding 3,5- and 3,7-diglucosides. In addition, several anthocyanin-derived wine pigments have identical masses and primary fragmentation patterns, such as the vinylformic acid adduct of cyanidin-3-glucoside and the acetaldehyde adduct of mavidin-3-glucoside (*m/z* 517, loss of 162), the 4-vinylguaicol adduct of peonidin-coumaroylglucoside and the 4-vinylphenol adduct of malvidin-coumaroylglucoside (*m/z* 755, loss of 308), and the 4-vinylcatechol adduct of malvidin-glucoside and the 4-vinylguaicol adduct of petunidin-glucoside (*m/z* 609, loss of 162), etc. (see further). This renders these compounds indistinguishable by MS alone. Since these isobaric compounds can be separated chromatographically, RP-LC in combination with MS offers a much more accurate technique for the detailed analysis of anthocyanins.

The chromatographic method used here is based on the optimal kinetic configuration for the RP-LC analysis of anthocyanins [16,17]. Due to the unique chromatographic behaviour of anthocyanins [16], very low optimal linear velocities are required for improved chromatographic separation of these molecules. By using a column packed with 1.7 µm particles operated at elevated temperature and the optimal flow rate for anthocyanins, highly efficient analysis of these compounds within reasonably short analysis times is possible [17] (see also Figure 8.2).

LC-MS in scan mode has been used extensively for the screening of wine anthocyanins, although compound identification is often tentatively based on elution order and molecular ion information only [17,19,20]. On the other hand, MS/MS is commonly used for highly selective and sensitive detection of a limited number of target analytes only [21,22].

Glycosylated anthocyanins and their derivatives are known to fragment *via* the loss of the dehydrated sugar moiety [1,14]. This phenomenon may be exploited to allow highly selective detection of these compounds by MS/MS in neutral loss scanning mode. This mode involves both quadrupole analysers being scanned with a preset mass offset so that when a target primary ion loses the predetermined mass, a signal is generated to detect that compound exclusively. Monitoring selected neutral losses in this manner allows the selective detection of anthocyanin-glycosides (neutral loss *m/z* 162), -diglucosides (neutral loss *m/z* 324), -acetylglucosides (neutral loss *m/z* 204), -coumaroylglucosides (neutral loss *m/z* 308) and caffeoylglucosides (neutral loss *m/z* 324), as well as the corresponding derived condensation products formed during wine production. The highly selective nature of neutral loss MS/MS experiments simplifies chromatographic analysis and facilitates tentative

compound identification utilising RP-LC chromatographic elution order. The neutral loss experiment also yields information pertaining to the molecular mass and mass of sugar moiety of the detected compounds. However, as highlighted above, the fact that various wine constituents have identical molecular ion masses and fragmentation patterns means that unambiguous compound identification is not guaranteed using this approach.

By performing a second experiment in survey scan mode, the cationic aglycone of each detected compound may be identified by its characteristic fragmentation pattern, as described by Hayasaka and Asenstrofer [14]. In survey scan mode, the instrument performs neutral loss scanning and switches to product ion mode at a specified ion count to fragment the target ion. The combination of chromatographic separation with MS/MS detection in neutral loss mode, combined with characterisation of the relevant aglycone by means of product ion spectra, significantly enhances the certainty of compound identification. This is especially relevant for wine pigments because standards for these compounds are not commercially available, and therefore comparison of retention time and mass spectral data with authentic standards is not possible. Using this approach, it was possible to identify 121 anthocyanins, pyranoanthocyanins and anthocyanin-flavanol adducts in Pinotage wines. The identification of each of these classes of compounds is discussed categorically in terms of chromatographic and MS/MS information in the following sections (8.3.1-8.3.3).

8.3.1. Anthocyanins

Figure 8.1 shows the fragmentation patterns for anthocyanins under low energy collision induced dissociation (CID) conditions. The nomenclature used here to discuss the fragmentation is in accordance with the adapted approach reported by Abad-García *et al*. [1]: $Y^{a,b}{}_{c}$ indicates the aglycone ion, where the glycosidic bond is specified by the subscript c, (i.e. 0 for the linkage with the aglycone, 1 for the inter-glycosidic bond of diglycosides, etc.). The position of glycosylation is denoted by superscripts a and b. Under high collision energies, rupture of the C-ring bonds results in charged fragments containing the A- and B-rings, denoted $[^{c,d}A]^{+}$ and $[^{c,d}B]^{+}$, respectively, where the position of the C-ring fragmentation is specified by the superscripts c and d (see also Figure 8.4).

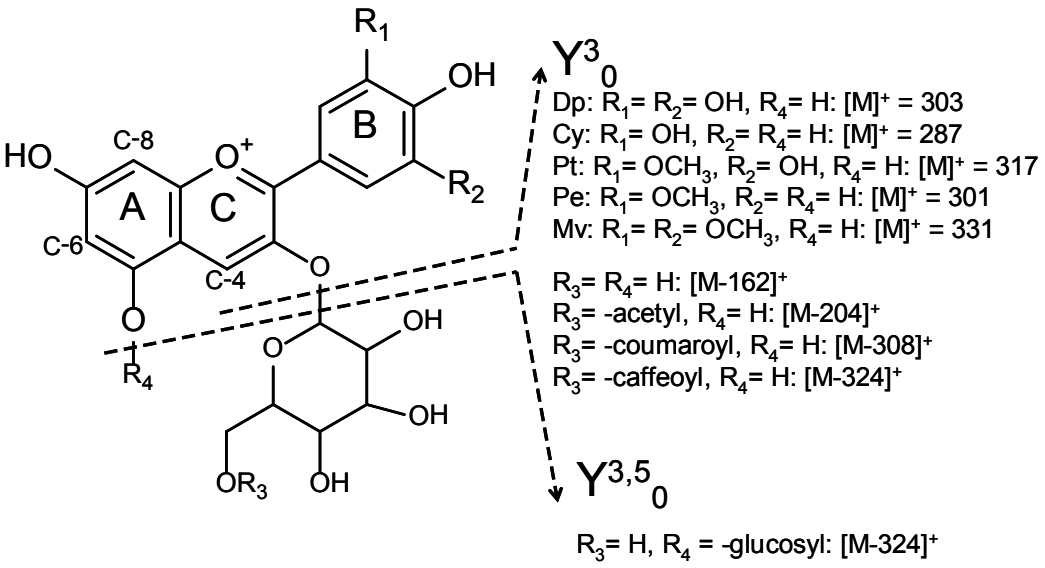


Figure 8.1. Neutral loss fragmentation of the anthocyanins and derived products under low energy CID conditions (25 V). Dp = delphinidin, Cy = cyanidin, Pt = petunidin, Pe = peonidin, Mv = malvidin.

Wine anthocyanins comprise mainly the anthocyanidin-monoglucosides and their acetyl-, *p*-coumaroyl- and caffeoyl- derivatives, as well as two groups of di-glucosides. Table 8.1 summarises the chromatographic and mass spectral parameters of the anthocyanins detected in Pinotage wines. These compounds display a structured elution pattern based on the characteristics of the anthocyanidin base [1]. The neutral loss chromatograms obtained for 162 amu (3-O-monoglucosides), 204 amu (3-O-acetylglucosides), 308 amu (3-O-coumaroylglucosides) and 324 amu (diglucosides and 3-O-caffeoylglucosides) (Figure 8.2) clearly demonstrate this structured elution order. Within each group, the elution order is: delphinidin < cyanidin < petunidin < peonidin < malvidin. This elution order is a function of the polarity of attached functional groups to the anthocyanidin base (hydroxyl groups decrease retention whereas methoxyl groups increase retention under RP-LC conditions). The acylated anthocyanins elute after the anthocyanins, while retention increase with hydrophobicity. Therefore the elution order is 3-O-monoglucosides < 3-O-acetylglucosides < 3-O-coumaroylglucosides, while the 3-O-caffeoylglucosides eluted among the 3-O-acetylglucosides. This characteristic elution order may be used to tentatively assign peaks based on their retention times relative to confirmed compounds.

Clear chromatographic separation was obtained between the *cis* and *trans* isomers of the 3-O-coumaroylglucosides. The neutral loss chromatogram (extracted ion at *m/z* = 639) of the isomers of malvidin-3-O-coumaroylglucoside is shown in Figure 8.2C. The *cis*-isomers of the 3-O-caffeoylglucosides [13] were not detected in any of the wines analysed here.

Table 8.1. Chromatographic and mass spectrometric parameters of detected anthocyanidin-glucosides, -diglucosides and oligomeric anthocyanins.

Peak no [a]	Compound [b]	Retention time (min)	M+ (*m/z*)	Neutral loss offset (amu)
13	Dp-3-O-glucoside	9.29	465	162
16	Cy-3-O-glucoside	11.00	449	162
23	Pt-3-O-glucoside	12.12	479	162
34	Pe-3-O-glucoside	13.97	463	162
41	Mv-3-O-glucoside	14.75	493	162
42	Dp-3-O-acetylglucoside	15.08	507	204
52	Cy-3-O-acetylglucoside	17.02	491	204
60	Pt-3-O-acetylglucoside	17.84	521	204
71	Pe-3-O-acetylglucoside	19.72	505	204
75	Mv-3-O-acetylglucoside	20.16	535	204
68	Dp-3-O-coumaroylglucoside (*cis*-isomer)	18.87 [c]	611	308
69	Dp-3-O-coumaroylglucoside (*trans*-isomer)	18.99 [c]	611	308
79	Cy-3-O-coumaroylglucoside (*cis*-isomer)	20.37	595	308
81	Cy-3-O-coumaroylglucoside (*trans*-isomer)	20.49	595	308
86	Pt-3-O-coumaroylglucoside (*cis*-isomer)	20.78	625	308
87	Pt-3-O-coumaroylglucoside (*trans*-isomer)	20.91	625	308
90	Pe-3-O-coumaroylglucoside (*cis*-isomer)	21.51	609	308
97	Pe-3-O-coumaroylglucoside (*trans*-isomer)	22.27	609	308
92	Mv-3-O-coumaroylglucoside (*cis*-isomer)	21.76	639	308
98	Mv-3-O-coumaroylglucoside (*trans*-isomer)	22.48	639	308
56	Dp-3-O-caffeoylglucoside	17.26 [c]	627	324
66	Pt-3-O-caffeoylglucoside	18.85	641	324
80	Pe-3-O-caffeoylglucoside	20.37 [c]	625	324
83	Mv-3-O-caffeoylglucoside	20.65 [c]	655	324
9	Dp-3,5-di-O-glucoside	7.93 [c]	627	324
18	Pt-3,5-di-O-glucoside	11.08 [c]	641	324
21	Pe-3,5-di-O-glucoside	11.76 [c]	625	324
25	Mv-3,5-di-O-glucoside	12.36 [c]	655	324
10	Dp-3,7-di-O-glucoside	8.70 [c]	627	324
24	Pt-3,7-di-O-glucoside	12.28 [c]	641	324
26	Pe-3,7-di-O-glucoside	12.73	625	324
30	Mv-3,7-di-O-glucoside	13.51	655	324
35	Pe-5,7-di-O-glucoside	14.06 [c]	625	324
45	Mv-5,7-di-O-glucoside	15.13	655	324
12	Dp-3-O-galactoside	9.09	465	162
33	Pe-3-O-galactoside	13.84	463	162
39	Mv-3-O-galactoside	14.45	493	162
61	Mv-Mv-2-O-glucoside dimer	17.85 [c]	985	162/324
76	Mv-O-glucoside-Mv-O-acetylglucoside dimer	20.18 [c]	1027	204

[a] Peak numbers correspond to Figure 8.2.
[b] Dp = delphinidin, Cy = cyanidin, Pt = petunidin, Pe = peonidin, Mv = malvidin.
[c] Survey scan spectra do not allow conclusive identification of aglycone due to low signal intensity.

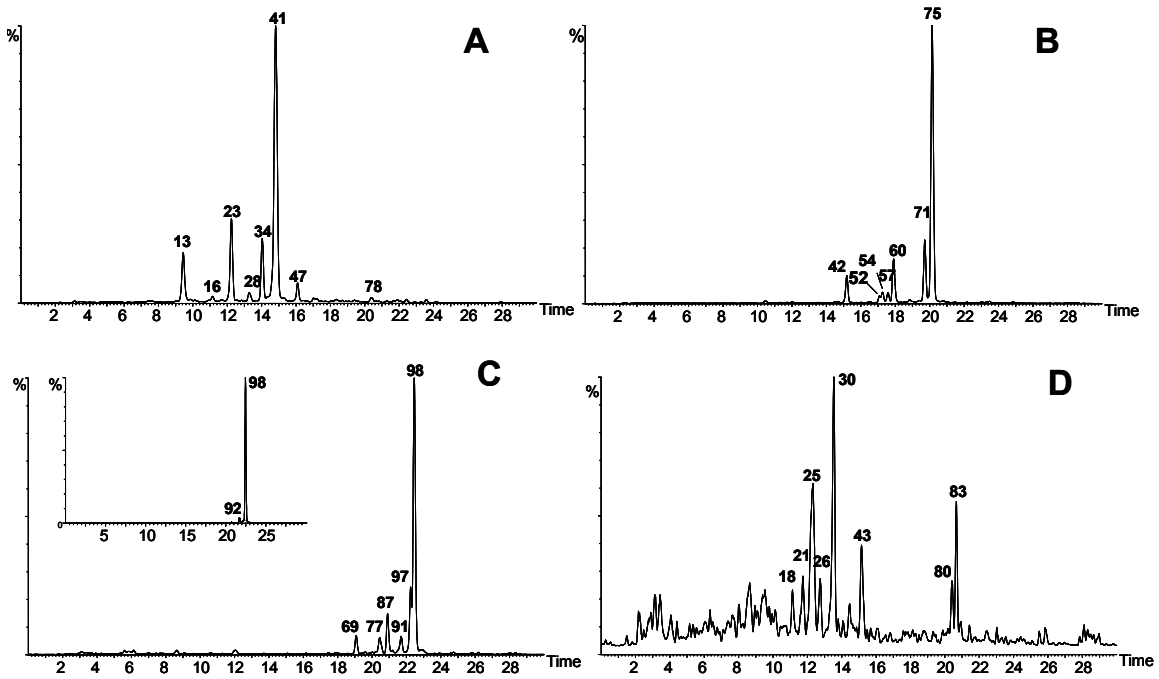


Figure 8.2. TICs of the LC-MS/MS neutral loss experiments recorded at (A) 162 amu (elimination of glucose), (B) 204 amu (elimination of acetylglucose), (C) 308 amu (elimination of *p*-coumaroylglucose) and (D) 324 amu (elimination of caffeoylglucose as well as diglucosides) for the analysis of a 2010 Pinotage wine. Insert (2C): Neutral loss chromatogram (308 amu) showing the isomers of malvidin-3-O-coumaroylglucoside (extracted ion at *m/z* = 639, *cis*-isomer at 21.46 minutes and *trans*-isomer at 22.48 minutes). Peak numbers correspond to Tables 8.1 and 8.3.

Two groups of anthocyanidin-diglucosides detected in this *Vitis vinifera* variety wine eluted before their corresponding anthocyanidin-monoglucosides, with the 3,5-diglucosides eluting before the 3,7-diglucoside isomeric forms, as also described in literature [13]. The presence of these compounds was confirmed by the analysis of the non-*Vitis vinifera* variety Dornfelder, which contains higher levels of diglucosides (results not shown). Three anthocyanidin-galactosides were also observed in the studied wines; these displayed identical fragmentation patterns compared to the corresponding glucoside isomers, but eluted earlier in the RP-LC separation [23,24]. Furthermore, both dimeric anthocyanins di-malvidin-diglucoside and malvidin-glucoside-malvidin-acetyl-glucoside [25-27] were detected in neutral loss mode (observed in traces for the loss of glucosides at 162 and 324 and acetyl-glucoside at 204, respectively). Oligomeric anthocyanins have been identified in grape skins [27], red wine [13,17] and grape extracts [26]. These compounds are characterised by very broad peaks due to the effect of secondary equilibria involving the anthocyanins [17].

The identities of the compounds detected in neutral loss mode were further confirmed by means of the relevant aglycone cation spectrum acquired in survey scan mode. Figure 8.3 shows the product ion spectra obtained for the five wine anthocyanidin-monoglucosides, while Table 8.2 lists the most prominent ions and their intensities. The fragments reported in Table 8.2 were confirmed by accurate mass measurements on a QTOF instrument (Supplementary Information, Table S8.1). Accurate mass measurements of the aglycone fragments all showed better than 5 ppm mass difference from the theoretical molecular formula values for the fragments ([1], Table 8.1, Table S8.1). These product ion spectra were consistent for all monoglucosides, irrespective of the modification to the sugar moieties, and were used to identify the aglycone cation for each of the anthocyanidin-monoglucosides in Table 8.1.

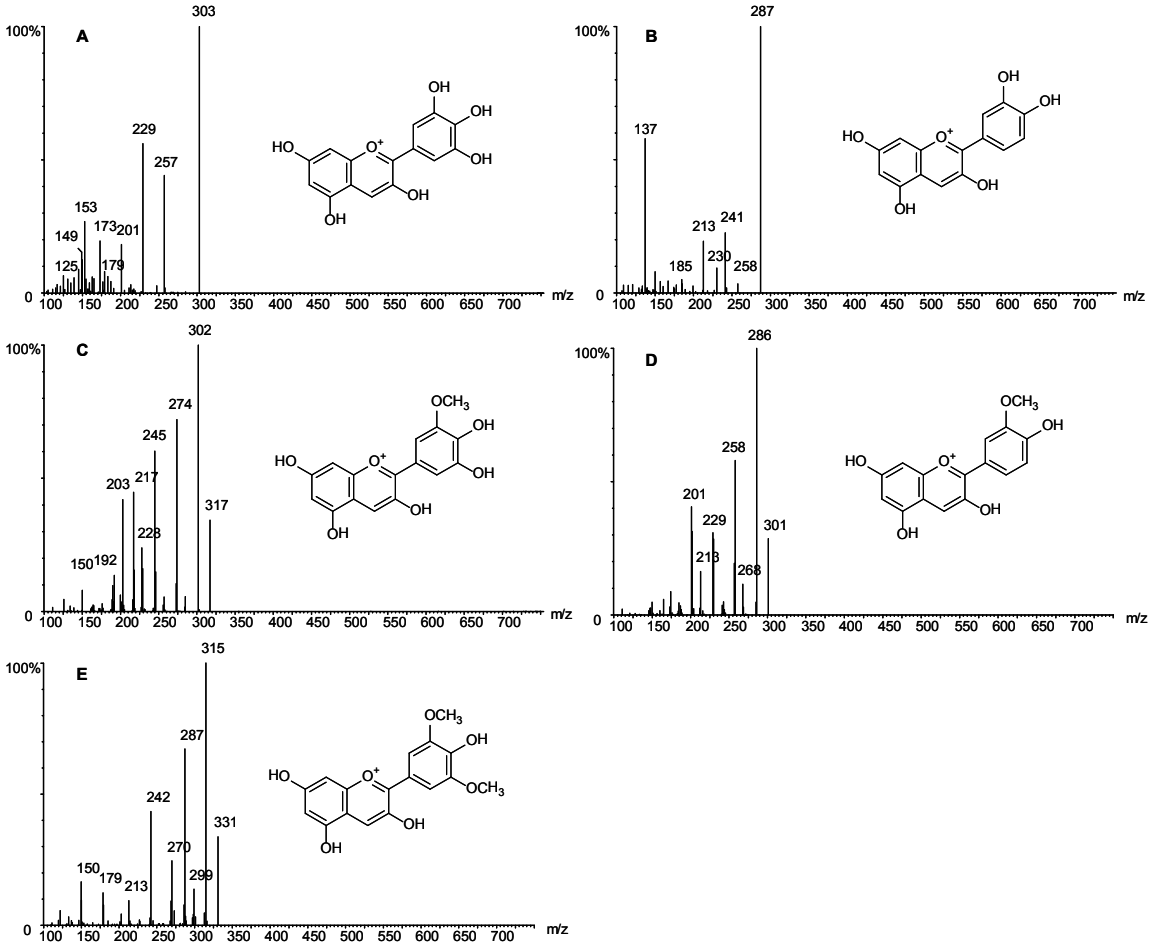


Figure 8.3. Characteristic product ion mass spectra of the anthocyanidin bases obtained in survey scan mode (concurrently with detection of the 3-O-monoglucoside anthocyanins in neutral loss mode at 162 amu) for (A) delphinidin, (B) cyanidin, (C) petunidin, (D) peonidin and (E) malvidin.

Table 8.2. Characteristic product ion spectra (*m/z*) obtained for the five anthocyanidin bases (% relative intensity in parenthesis).

Product ions	delphinidin	cyanidin	petunidin	peonidin	malvidin
[M]+	303(100)	287(100)	317(46)	301(27)	331(23)
[M-CH_3]+•			302(100)	286(100)	
[M-(CH_3+H)]+					315(100)
[M-CH_3OH]+					299(24)
[M-(H+CH_3OH)]+•				268(12)	
[M-CH_3-CO]+•			274(53)	258(62)	
[M-CH_3-(H+CO)]+					287(51)
[M-CH_3OH-(H+CO)]+•					270(24)
[M-CH_3-2CO)]+•				230(29)	
[M-CH_3-CO-(H+CO)]+			245(76)	229(30)	
[M-CH_3OH-2CO]+				213(15)	
[M-CH_3-3CO)]+•				202(32)	
[M-CH_3-2CO-(H+CO)]+			217(29)	201(38)	
[M-CH_3OH-(H+CO)-CO]+•			228(22)		242(51)
[M-CH_3-CO-(H+CO)-C_2H_2O]+			203(43)		
[M-(H+CO)]+•		258(4)			
[M-CH_3OH-2(H+CO)-CO]+					213(10)
[M-H_2O-CO]+	257(33)	241(22)			
[M-(H+CO)-CO]+•		230(10)			
[M-H_2O-2CO]+	229(48)	213(20)			
[M-H_2O-3CO]+	201(12)	185(5)			
[M-B-ring]+	179(8)				179(18)
[M-H_2O-4CO]+	173(20)				
[$^{0,2}A_0$]+•	150(13)	150(8)	150(9)	150(5)	150(17)
[$^{0,2}A_0$]+	149(16)				
[$^{0,2}B_0$]+	153(27)	137(59)			
[$^{0,3}A_0$]+		120(3)			
[$^{0,2}B_0$-CO]+	125(7)				

Some of the major fragmentation patterns observed for the anthocyanidins are illustrated in Figure 8.4 In addition to the indicated fragmentation pathways, the product ion spectra also displayed fragments corresponding to the loss of CH_3, CH_3OH, CO and/or H atoms or radicals from the respective anthocyanidins. These fragmentation processes generally predominate over fragmentation related to rupture of the C-ring bonds (see Figure 8.4 and Table 8.2). The product ion spectra of hydroxylated anthocyanins (cyanidin and delphinidin) are characterised by the molecular ion (aglycone cation) as the base peak, while the methoxylated anthocyanins (malvidin, petunidin and peonidin) also show relatively intense peaks for the molecular ion. The base peaks in the spectra for petunidin and peonidin result from the loss of a methyl group, while the base peak in the spectrum of malvidin results from the simultaneous elimination of a methyl group and a proton. All five wine anthocyanidins yielded the $[^{0,2}A_0]^{+\bullet}$ radical cation (*m/z* = 150) resulting from cleavage of the 0/2 C-ring bonds (Figure 8.4). The presence of this fragment in the product ion spectra may be ascribed to resonance stabilisation of the formed $[^{0,2}A_0]^{+\bullet}$ radical [1]. Only delphinidin yields the $[^{0,2}A_0]^{+}$ cation (*m/z* = 149). A similar cleavage of the 0/2 C-ring also results in the corresponding

$[^{0,2}B_0]^+$ ion, yielding fragments at *m/z* = 153 and 137 for delphinidin and cyanidin, respectively. Delphinidin displays a fragment due to the further loss of CO from the $[^{0,2}B_0]^+$ ion to yield $[^{0,2}B_0\text{-CO}]^+$ (*m/z* = 125). Malvidin and cyanidin show fragments at *m/z* = 179 due the loss of the B-ring, while cyanidin has a weak fragment corresponding to the $[^{0,3}A_0]^+$ ion at *m/z* = 120.

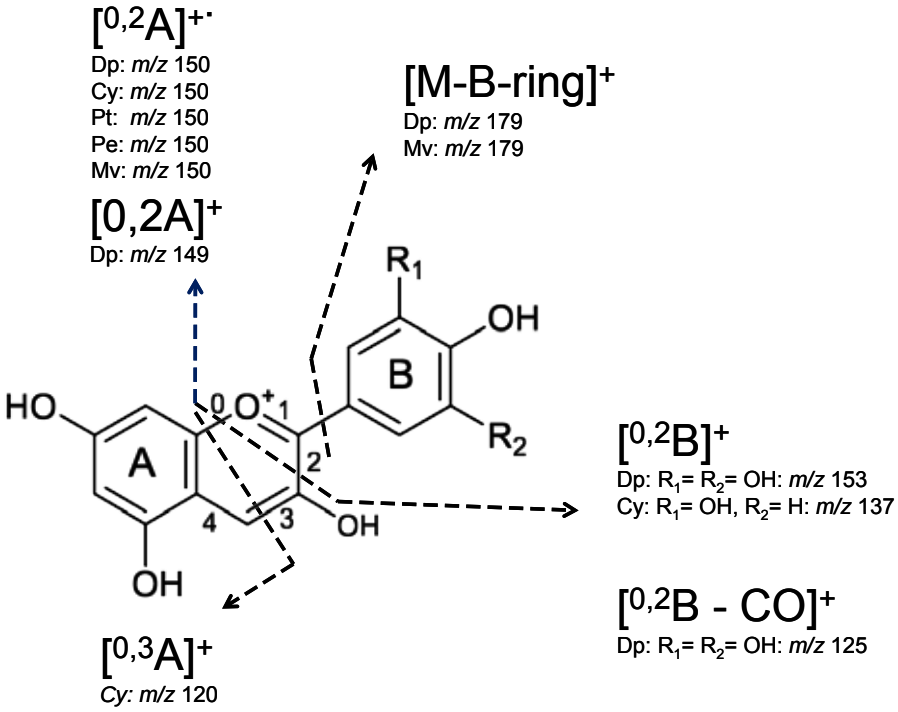


Figure 8.4. Typical fragmentation involving the C-ring for anthocyanidin aglycone cations under high collision energies (Table 8.1). Dp = delphinidin, Cy = cyanidin, Pt = petunidin, Pe = peonidin, Mv = malvidin.

The anthocyanidin-diglucosides displayed aglycone cation fragmentation spectra that contained identical fragments compared to the corresponding anthocyanidin-glucosides, but present at different relative intensities. This phenomenon may be a result of the presence of the substituted sugar on the 5 and 7 positions of the A-ring, which affects fragmentation of these molecules. Figure 8.5 shows the product ion spectra obtained for the two groups of malvidin-diglucosides.

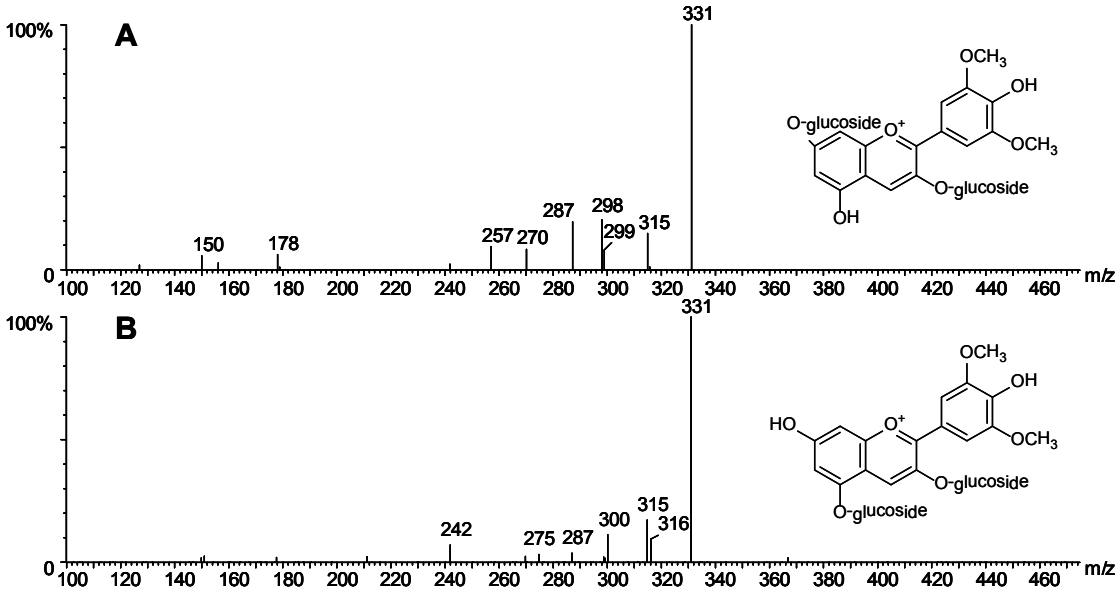


Figure 8.5. Characteristic product ion mass spectra and structures of (A) malvidin-3,7-di-O-glucoside (peak 45, Table 8.1) and (B) malvidin-3,5-di-O-glucoside (peak 30, Table 8.1). Note the differing intensities of the ions compared to Figure 8.3 (E) for malvidin-3-O-glucoside.

8.3.2. Pyranoanthocyanins

Some wine constituents possessing a polarisable double bond react with the anthocyanins (monoglucosides as well as acylated derivatives) to induce cyclysation between C-4 and the hydroxyl on C-5 of the anthocyanidin. Compounds such as pyruvic acid [9,10], acetaldehyde [11], acetone [28], 4-vinylcatechol [29], 4-vinylphenol [12], 4-vinylguaiacol [14] and vinylcatechin participate in these cyclo-addition reactions [3,13,14]. In the case of 4-vinylphenol, 4-vinylguaiacol, 4-vinylcatechol and 4-vinylsyringol, adduct formation is the result of the reaction between the relevant hydroxycinnamic acids (coumaric acid, ferulic acid, caffeic acid and synaptic acid, respectively) and the anthocyanin [30]. Subsequent decarboxylation and oxidation of the intermediate carbenium ion leads to the formation of these pyranoanthocyanins [30].

Importantly, the pyranoanthocyanins are also detected selectively in neutral loss scanning mode *via* elimination of the relevant glycosyl moieties as described above for the anthocyanins [14]. Table 8.3 summarises the chromatographic and mass spectral parameters for the pyranoanthocyanins detected in the Pinotage wines under investigation. Retention of pyranoanthocyanins in RP-LC increased with increasing hydrophobicity of the adduct, with the following elution order: vinylformic acid < acetaldehyde < acetone < 4-

vinylcatechol < 4-vinylphenol < 4-vinylguaiacol. Within each class, the elution order was the same as observed for the unmodified anthocyanins.

Significantly, the mass spectra of the aglycone cations of pyranoanthocyanins obtained in survey scan mode are characterised by the elimination of identical fragments compared to the corresponding unmodified anthocyanins [14]. This is because modification to the anthocyanidin base occurs between C-4 and C-5. Since the primary fragmentation of anthocyanidins involves cleavage of the bonds between C-1 and C-4 (Figure 8.4), identical losses are also observed for the pyranoanthocyanins (the exception being for the $[^{0,3}A]^+$ fragment of cyanidin-glucoside). This observation also confirms that the loss of CH_3, CH_3OH, CO and H fragments occurs at the B-ring of the anthocyanidins.

This fragmentation behaviour means that for the pyranoanthocyanidins the fragment ions obtained are offset by a consistent mass compared to the corresponding parent anthocyanidin, with the offset determined by the mass of the relevant adduct. For example, the fragments in the product ion spectra of the vinylformic acid adducts are offset by 68 amu compared to the characteristic fragment ions of the unmodified anthocyanidin base. Similarly, for the acetaldehyde, acetone, 4-vinylcatechol, 4-vinylphenol and 4-vinylguaiacol derivatives this offset corresponds to 24, 38, 132, 116 and 146 amu, respectively. The pyranoanthocyanins may therefore also be identified by applying the criteria outlined above for the anthocyanins. Figure 8.6 shows the characteristic aglycone cation mass spectra obtained for malvidin-3-O-glucoside congeners of selected pyranoanthocyanins. It is important to note, however, that the relative intensities of some of the characteristic fragments are different compared to malvidin-3-O-glucoside (Figure 8.3 (E)). The vinylformic acid-, acetaldehyde-, acetone-, 4-vinylcatechol-, 4-vinylphenol- and 4-vinylguaiacol- adducts of anthocyanins and their acylated derivatives were identified (Table 8.3).

Table 8.3. Chromatographic and mass spectrometric parameters of detected pyranoanthocyanins and anthocyanin-flavonol condensation products.

Peak no [a]	Compound [b]	Retention time (min)	M+ (*m/z*)	Neutral loss offset (amu)
15	Vinylformic acid adduct of Dp-3-O-glucoside	9.74 [c]	533	162
22	Vinylformic acid adduct of Cy-3-O-glucoside	12.00	517	162
28	Vinylformic acid adduct of Pt-3-O-glucoside	13.10	547	162
46	Vinylformic acid adduct of Pe-3-O-glucoside	15.17 [c]	531	162
47	Vinylformic acid adduct of Mv-3-O-glucoside	15.99	561	162
17	Vinylformic acid adduct of Dp-3-O-acetylglucoside	11.04 [c]	575	204
32	Vinylformic acid adduct of Cy-3-O-acetylglucoside	13.75 [c]	559	204
38	Vinylformic acid adduct of Pt-3-O-acetylglucoside	14.33 [c]	589	204
54	Vinylformic acid adduct of Pe-3-O-acetylglucoside	17.07 [c]	573	204
57	Vinylformic acid adduct of Mv-3-O-acetylglucoside	17.46	603	204
44	Vinylformic acid adduct of Dp-3-O-coumaroylglucoside	15.12 [c]	679	308
51	Vinylformic acid adduct of Cy-3-O-coumaroylglucoside	16.90 [c]	663	308
58	Vinylformic acid adduct of Pt-3-O-coumaroylglucoside	17.80 [c]	693	308
74	Vinylformic acid adduct of Pe-3-O-coumaroylglucoside	20.12 c	677	308
77	Vinylformic acid adduct of Mv-3-O-coumaroylglucoside	20.34	707	308
43	Vinylformic acid adduct of Mv-3-O-caffeoylglucoside	15.11	723	324
19	Acetaldehyde adduct of Dp-3-O-glucoside	11.11	489	162
27	Acetaldehyde adduct of Cy-3-O-glucoside	13.04	473	162
36	Acetaldehyde adduct of Pt-3-O-glucoside	14.29	503	162
50	Acetaldehyde adduct of Pe-3-O-glucoside	16.17	487	162
53	Acetaldehyde adduct of Mv-3-O-glucoside	17.03	517	162
29	Acetaldehyde adduct of Dp-3-O-acetylglucoside	13.13 [c]	531	204
48	Acetaldehyde adduct of Pt-3-O-acetylglucoside	16.09 [c]	545	204
64	Acetaldehyde adduct of Pe-3-O-acetylglucoside	18.40 [c]	529	204
67	Acetaldehyde adduct of Mv-3-O-acetylglucoside	18.85	559	204
59	Acetaldehyde adduct of Dp-3-O-coumaroylglucoside	17.81 [c]	635	308
89	Acetaldehyde adduct of Pe-3-O-coumaroylglucoside	21.45 [c]	633	308
91	Acetaldehyde adduct of Mv-3-O-coumaroylglucoside	21.66 [c]	663	308
37	Acetone adduct of Dp-3-O-glucoside	14.29	503	162
49	Acetone adduct of Cy-3-O-glucoside	16.13 [c]	487	162
55	Acetone adduct of Pt-3-O-glucoside	17.08 [c]	517	162
65	Acetone adduct of Pe-3-O-glucoside	18.50 [c]	501	162
70	Acetone adduct of Mv-3-O-glucoside	19.18 [c]	531	162
85	Acetone adduct of Mv-3-O-acetylglucoside	20.70	573	204
73	Acetone adduct of Dp-3-O-coumaroylglucoside	20.03 [c]	649	308
102	Acetone adduct of Pe-3-O-coumaroylglucoside	22.90 [c]	647	308
103	Acetone adduct of Mv-3-O-coumaroylglucoside	23.17 [c]	677	308
62	4-Vinylcatechol adduct of Dp-3-O-glucoside	18.19	597	162
82	4-Vinylcatechol adduct of Pt-3-O-glucoside	20.61	611	162
96	4-Vinylcatechol adduct of Pe-3-O-glucoside	22.19 [c]	595	162
99	4-Vinylcatechol adduct of Mv-3-O-glucoside	22.51	625	162
106	4-Vinylcatechol adduct of Pe-3-O-acetylglucoside	23.45 [c]	637	204
107	4-Vinylcatechol adduct of Mv-3-O-acetylglucoside	23.55 [c]	667	204

101	4-Vinylcatechol adduct of Pt-3-O-coumaroylglucoside	22.88 [c]	757	308
111	4-Vinylcatechol adduct of Pe-3-O-coumaroylglucoside	24.58 [c]	741	308
112	4-Vinylcatechol adduct of Mv-3-O-coumaroylglucoside	24.74 [c]	771	308
72	4-Vinylphenol adduct of Dp-3-O-glucoside	19.84 [c]	581	162
78	4-Vinylphenol adduct of Cy-3-O-glucoside	20.34 [c]	565	162
94	4-Vinylphenol adduct of Pt-3-O-glucoside	21.99 [c]	595	162
104	4-Vinylphenol adduct of Pe-3-O-glucoside	23.35 [c]	579	162
108	4-Vinylphenol adduct of Mv-3-O-glucoside	23.68	609	162
88	4-Vinylphenol adduct of Dp-3-O-acetylglucoside	20.95 [c]	623	204
105	4-Vinylphenol adduct of Pt-3-O-acetylglucoside	23.41 [c]	637	204
113	4-Vinylphenol adduct of Pe-3-O-acetylglucoside	24.75	621	204
114	4-Vinylphenol adduct of Mv-3-O-acetylglucoside	24.98	651	204
118	4-Vinylphenol adduct of Pe-3-O-coumaroylglucoside	26.19 [c]	725	308
119	4-Vinylphenol adduct of Mv-3-O-coumaroylglucoside	26.30	755	308
84	4-Vinylguaiacol adduct of Dp-3-O-glucoside	20.66 [c]	611	162
95	4-Vinylguaiacol adduct of Cy-3-O-glucoside	22.16 [c]	595	162
100	4-Vinylguaiacol adduct of Pt-3-O-glucoside	22.66 [c]	625	162
109	4-Vinylguaiacol adduct of Pe-3-O-glucoside	23.89 [c]	609	162
110	4-Vinylguaiacol adduct of Mv-3-O-glucoside	24.29	639	162
93	4-Vinylguaiacol adduct of Dp-3-O-acetylglucoside	21.83 [c]	653	204
116	4-Vinylguaiacol adduct of Pe-3-O-acetylglucoside	25.33 [c]	651	204
117	4-Vinylguaiacol adduct of Mv-3-O-acetylglucoside	25.59 [c]	681	204
115	4-Vinylguaiacol adduct of Pt-3-O-coumaroylglucoside	25.04 [c]	771	308
120	4-Vinylguaiacol adduct of Pe-3-O-coumaroylglucoside	26.43 [c]	755	308
121	4-Vinylguaiacol adduct of Mv-3-O-coumaroylglucoside	26.83 [c]	785	308
6	Catechin adduct of Dp-3-O-glucoside (T-A)	5.40 [c]	753	162
8	Catechin adduct of Pt-3-O-glucoside (T-A)	6.96 [c]	767	162
11	Catechin adduct of Pe-3-O-glucoside (T-A)	8.77 [c]	751	162
14	Catechin adduct of Mv-3-O-glucoside (T-A)	9.43 [c]	781	162
31	Catechin adduct of Pe-3-O-acetylglucoside	13.69 [c]	793	204
40	Catechin adduct of Mv-3-O-acetylglucoside	14.58 [c]	823	204
63	Catechin adduct of Mv-3-O-coumaroylglucoside	18.26 [c]	927	308
20	Epi-catechin adduct of Mv-3-O-glucoside (T-A)	11.50 [c]	781	162
1	Gallocatechin adduct of Pt-3-O-glucoside (T-A)	3.05 [c]	783	162
2	Gallocatechin adduct of Pe-3-O-glucoside (T-A)	3.51 [c]	767	162
3	Gallocatechin adduct of Mv-3-O-glucoside (T-A)	4.11 [c]	797	162
4	Epi-gallocatechin adduct of Pt-3-O-glucoside (T-A)	4.58 [c]	783	162
5	Epi-gallocatechin adduct of Pe-3-O-glucoside (T-A)	5.19 [c]	767	162
7	Epi-gallocatechin adduct of Mv-3-O-glucoside (T-A)	5.79 [c]	797	162

[a] Peak numbers correspond to Figure 8.2.
[b] Dp = delphinidin, Cy = cyanidin, Pt = petunidin, Pe = peonidin, Mv = malvidin.
[c] Survey scan spectra do not allow conclusive identification of aglycone due to low signal intensity.

8.3.3. Direct and acetaldehyde-mediated anthocyanin-flavanol condensation products

Several proanthocyanidin-anthocyanin adducts were also detected in neutral loss scanning mode. These compounds are formed by reaction of procyanidins and anthocyanins *via* coupling between C-4 of the anthocyanidin and either C-6 or C-8 of an (epi)catechin moiety of the procyanidin molecule (referred to as A-T condensation products) or between C-6 or C-8 of the anthocyanidin and C-6 or C-8 of the procyanidin (T-A condensation) [4,6,31]. The latter compounds occur in the bicyclic form [31,32] and elute before the corresponding A-T products under RP-LC conditions [17]. Only the T-A type anthocyanin-flavanol adducts containing one catechin or epicatechin unit were detected in the Pinotage wines investigated here (Table 8.3). The fact that no A-T condensation products or higher molecular weight T-A adducts were detected might be due to additional fragmentation patterns involving the procyanidin moiety, such as loss of (epi)catechin (*m/z* 288) and retro-Diels-Alder fission of the (epi)catechin units (*m/z* 152) [17]. In fact, additional experiments were performed in neutral loss mode by monitoring these losses, but these only allowed detection of free procyanidins in the wines studied. However, since especially the higher molecular weight anthocyanin-procyanidin adducts are present in relatively low concentrations, the additional fragmentation pathways involving these molecules (which are expected to become predominant with increase in the size of the procyanidin group) are presumably responsible for the fact that none of these compounds were detected in neutral loss mode utilising the characteristic losses for anthocyanins only.

Six prodelphinidin-anthocyanin adducts were also detected in neutral loss scanning mode. These compounds eluted before the corresponding procyanidin-anthocyanin adducts [17]. The peak shapes for both prodelphinidin- and procyanidin-anthocyanin adducts were relatively broad due to the effect of secondary equilibria involving the anthocyanin moiety of these compounds [17].

It is also worth noting that no vinylcatechin adducts of anthocyanins, where the anthocyanin is linked at the C-4 position *via* a vinyl linkage to a procyanidin, were detected in the current study [33], This may once again be due to the presence of additional fragmentation patterns of these molecules, which prohibit their detection at low concentrations using the neutral loss experiments performed here.

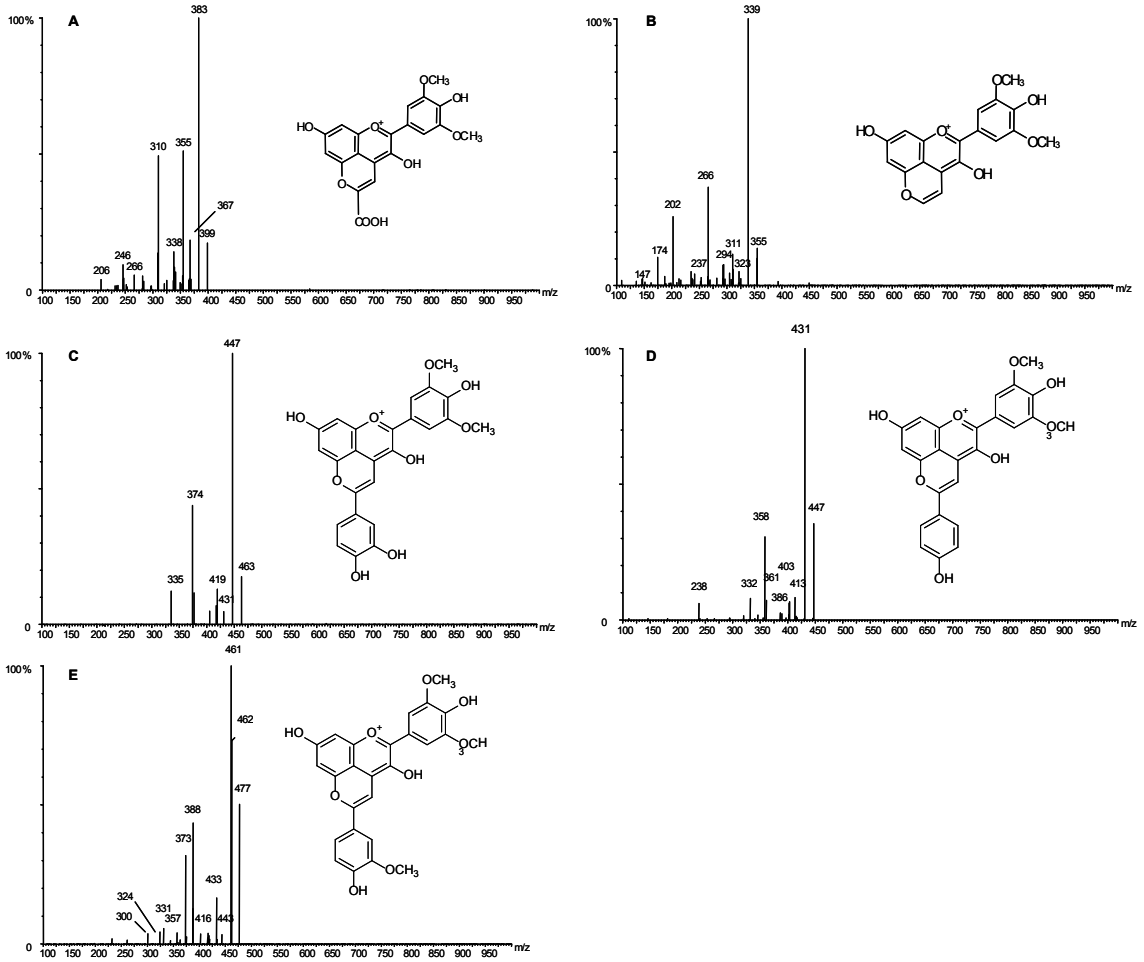


Figure 8.6. Characteristic survey scan product ion mass spectra and structures of the pyranoanthocyanin derivatives of malvidin-3-O-glucoside, (A) vinylformic acid adduct, (B) acetaldehyde adduct, (C) vinylcatechol adduct, (D) vinylphenol adduct and (E) vinylguaiacol adduct.

Note that the approach used here was developed specifically to exploit the benefits of triple quadrupole instrumentation. Alternative mass analysers can also be used advantageously for anthocyanin analysis. For example, high resolution TOF instruments provide the benefits of accurate mass for identification purposes, whereas QTOF instruments provide improved sensitivity in full scan mode and can be used to obtain accurate mass spectra of the daughter anthocyanidins. However, QTOF instruments cannot be operated in neutral loss mode, and are therefore not suited for the selective screening of wine anthocyanins in a similar manner as used in the current contribution. Nor can neutral-loss-directed survey scans be performed on such instrumentation. Therefore, while alternative mass analysers can beneficially be applied for anthocyanin analysis, a similar selective screening and identification approach as reported here requires the use of triple quadrupole (or ion-trap) instrumentation. Considering the relatively lower cost and more widespread availability of

triple quadrupole instruments, the method reported here shows promise for the detailed screening of pigmented fractions of wine.

8.4. Conclusions

A systematic approach for the detailed investigation of red wine pigments has been developed by exploiting the advantages inherent to optimised chromatographic separation in combination with the selective detection capabilities and structural identification power of MS/MS utilising a triple quadrupole instrument. The proposed approach involves two sets analyses: in the first instance, neutral loss scanning is performed for losses of 162, 204, 308 and 324 amu to selectively detect anthocyanin glucosides, diglucosides and acylated derivatives, as well as the corresponding derivatives formed during wine ageing. This is followed by four survey scan experiments to identify the aglycone cation based on characteristic fragmentation processes for the anthocyanidins. The highly selective nature of neutral loss detection simplifies the analysis, and in combination with of the structured RP-LC chromatographic elution order allows tentative compound identification. Moreover, neutral loss scanning reveals the molecular weight and mass of the attached sugar moiety, which further aids compound identification. Characterisation of the aglycone cation by a second set of experiments in survey scan mode ensures unambiguous identification of detected compounds. The highly selective nature of the neutral loss experiments also makes this mode of detection inherently suited to quantitative determination of these compounds. The limits of detection under our conditions are approximately 0.1 mg/L in neutral loss mode, while consistent product ion mass spectra were obtained in survey scan mode at concentrations above 5 mg/L (as determined for malvidin-3-O-glucoside). This methodology therefore offers a powerful and simple approach for the selective determination of the anthocyanins and derived compounds in red wines. This method allowed us to identify a total of 121 different compounds belonging to the anthocyanins, pyranoanthocyanins, and flavanol-anthocyanin condensation products in Pinotage wines. The primary benefits of this approach are two-fold: increased selectivity simplifies identification and minimises co-elution so that the structured RP-LC elution may be exploited to allow more accurate tentative compound identification. Secondly, product ion spectra significantly increase the certainty in identification (at least of major compounds). The MS/MS spectra reported here for the first time for anthocyanin derivatives in wine will prove useful in minimising the risks of false identification, which is always a factor due to the unavailability of standards for wine anthocyanins. The method therefore offers a simple and fast approach for qualitative screening of red wine anthocyanins where the use of RP-LC overcome some of the limitations of direct infusion MS such as ion suppression as well as wrong assignment of compounds due to identical masses of many wine pigments.

Further research is required to establish the suitability of the proposed methodology for quantitative analysis. This would allow more accurate and detailed investigation of the evolution of wine anthocyanins and their derived products during wine ageing.

References

[1] B. Abad-García, L.A. Berrueta, S. Garmón-Lobato, B. Gallo, F. Vicente, J. Chromatogr. A 1216 (2009) 5398.
[2] B. Berente, D.D. García, M. Reichenbächer, K. Danzer, J. Chromatogr. A 871 (2000) 95.
[3] R.S. Jackson, Wine science: Principles, practice and perception, Academic Press, San Diego, U.S.A. (2000).
[4] B.W. Zoecklein, K.C. Fugelsang, B.H. Gump, F.S. Nury, Production wine analysis, Chapman & Hall, New York, U.S.A. (1990).
[5] E.M. Francia-Aricha, M.T. Guarra, J.C. Rivas-Gonzalo, C. Santos-Buelga, J. Agric. Food Chem. 45 (1997) 2262.
[6] S. Remy, H. Fulcrand, B. Labarbe, V. Cheynier, M. Moutounet, J. Sci. Food Agric. 80 (2000) 745.
[7] J.C. Rivas-Gonzalo, S. Bravo-Haro, C. Santos-Buelga, J. Agric. Food Chem. 43 (1995) 1444.
[8] E. Salas, V. Atanasova, C. Poncet-Legrand, E. Meudec, J.P. Mazauric, V. Cheynier, Anal. Chim. Acta 513 (2004) 325.
[9] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutounet, Phytochem. 47 (1998) 1401.
[10] C. Romero, J. Bakker, J. Agric. Food Chem. 47 (1999) 3130.
[11] J. Bakker, C.F. Timberlake, J. Agric. Food Chem. 45 (1997) 35.
[12] H. Fulcrand, P.J. Cameira dos Santos, P. Sarni-Manchado, V. Cheynier, J. Favre-Bonvin, J. Chem. Soc. Perkin Trans. 1 (1996) 735.
[13] C. Alcalde-Eon, M.T. Escribano-Bailón, C. Santos-Buelga, J.C. Rivas-Gonzalo, Anal. Chim. Acta 563 (2006) 238.
[14] Y. Hayasaka, R.E. Asenstrofer, J. Agric. Food Chem. 50 (2002) 756.
[15] N. Mateus, A.M. Silva, J.C. Rivas-Gonzalo, C. Santos-Buelga, V. de Freitas, J. Agric. Food Chem. 51 (2003) 1919.
[16] A. de Villiers, D. Cabooter, F. Lynen, G. Desmet, P. Sandra, J. Chromatogr. A 1216 (2009) 3270.
[17] A. de Villiers, D. Cabooter, F. Lynen, G. Desmet, P. Sandra, J. Chromatogr. A 1218 (2011) 4660.
[18] D. Favretto, R. Flamini, Am. J. Enol. Vitic. 51 (2000) 55.
[19] V. Atanasova, H. Fulcrand, V. Cheynier, M. Moutounet, Anal. Chim. Acta 458 (2002) 15.
[20] A. de Villiers, G. Vanhoenacker, P. Majek, P. Sandra, J. Chromatogr. A 1054 (2004) 195.
[21] J.Y. Choi, S.J. Lee, S.J. Lee, S. Park, J.H. Lee, J.H. Shim, A.M.A. El-Aty, J.S. Jin, E.D. Jeong, W.S. Lee, S.C. Shin, J. Sep. Sci. 33 (2010) 1192.
[22] K. Nagy, K. Redeuil, R. Bertholet, H. Steiling, M. Kussmann, Anal. Chem. 81 (2009) 6347.
[23] L.G. Wade, Organic chemistry, Prentice-Hall Inc., Upper Saddle River, New Jersey, U.S.A. (1999).
[24] H. Wang, E.J. Race, A.J. Shrikhande, J. Agric. Food Chem. 51(2003) 1839.
[25] C. Alcalde-Eon, M.T. Escribano-Bailón, C. Santos-Buelga, J.C. Rivas-Gonzalo, J. Mass Spectrom. 42 (2007) 735.
[26] S. Pati, I. Losito, G. Gambacorta, E.L. Notte, F. Palmisano, P.G. Zambonin, J. Mass Spectrom. 41 (2006) 861.
[27] S. Vidal, E. Meudec, V. Cheynier, G. Skouroumounis, Y. Hayasaka, J. Agric. Food Chem. 52 (2004) 7144.
[28] Y. Lu, L.Y. Foo, Tetrahedron Lett. 42 (2001) 1371.
[29] A.E. Håkansson, K. Pardon, Y. Hayasaka, M. De Sa, M. Herderich, Tetrahedron Lett. 44 (2003) 4887.
[30] M. Schwarz, T.C. Wabnitz, P. Winterhalter, J. Agric. Food Chem. 51 (2003) 3682.

[31] M. Monagas, B. Bartolomé, C. Gómez-Cordovés, Food Science and Nutrition 45 (2005) 85.
[32] S. Remy-Tanneau, C. Le Guerneve, E. Meudec, V. Cheynier, J. Agric. Food Chem. 51 (2003) 3592.
[33] J. He, C. Santos-Buelga, N. Mateus, V. de Freitas, J. Chromatogr. A 1134 (2006) 215.

Supplementary information

Table S8.1. Accurate mass information for the fragments detected in the product ion spectra of the five anthocyanidin bases by UHPLC-QTOF-MS (% relative intensity in parenthesis).

Product ions	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin
[M]+	303.0507(100)	287.0553(100)	317.0664(46)	301.0710(27)	331.0815(23)
[M-CH_3]+•			302.0428(100)	286.0476(100)	
[M-(CH_3+H)]+					315.0504(100)
[M-CH_3OH]+					299.0550(24)
[M-(H+CH_3OH)]+•				268.0364(12)	
[M-CH_3-CO]+•			274.0473(53)	258.0520(62)	
[M-CH_3-(H+CO)]+					287.0549(51)
[M-CH_3OH-(H+CO)]+•					270.0527(24)
[M-CH_3-2CO)]+•				230.0574(29)	
[M-CH_3-CO-(H+CO)]+			245.0450(76)	229.0502(30)	
[M-CH_3OH-2CO]+				213.0569(15)	
[M-CH_3-3CO)]+•				202.0630(32)	
[M-CH_3-2CO-(H+CO)]+			217.0503(29)	201.0537(38)	
[M-CH_3OH-(H+CO)-CO]+•			228.0424(22)		242.0576(51)
[M-CH_3-CO-(H+CO)-C_2H_2O]+			203.0345(43)		
[M-(H+CO)]+•		258.0518(4)			
[M-CH_3OH-2(H+CO)-CO]+					213.0553(10)
[M-H_2O-CO]+	257.0449(33)	241.0506(22)			
[M-(H+CO)-CO]+•		230.0575(10)			
[M-H_2O-2CO]+	229.0501(48)	213.0547(20)			
[M-H_2O-3CO]+	201.0515(12)	185.0532(5)			
[M-B-ring]+	179.0346(8)				179.0343(18)
[$^{0,2}A_0$]+•	150.0317(13)	150.0298(8)			150.0316(17)
[$^{0,2}A_0$]+	149.0246(16)				
[$^{0,2}B_0$]+	153.0191(27)	137.0240(59)			
[$^{0,2}B_0$-CO]+	125.0602(7)				

Chapter 9

Summary, conclusions and perspectives

9.1. Summary

The global wine industry is an important sector of agriculture, and wine analysis forms the basis of assessing compliance of its products with standards for international trade, as well as for research purposes. Wine is a highly complex sample matrix that places extreme demands on analytical techniques. In particular, research in this field increasingly requires methods of analysis that are capable of higher sensitivity, selectivity and specificity.

Chromatography has found widespread application in wine analysis due to the inherent capability of the technique to separate complex mixtures and to quantify components. High performance liquid chromatography (HPLC) offers a rugged, versatile and selective analytical technique that is amenable to a wide range of analytes, and it is principally used for the analysis on non-volatile wine components. However, conventional, non-spectroscopic detectors used in combination with HPLC lack the sensitivity and specificity for the determination of especially trace level wine components, and in addition, do not posses spectral capabilities for compound identification or structure elucidation purposes. Hyphenation of mass spectrometry (MS) with HPLC has created a powerful set of analytical tools that combine the separation and quantitative capabilities of chromatography with the sensitivity and spectral capabilities inherent to MS. As a result, liquid chromatography – mass spectrometry (LC-MS) is increasingly being applied in the wine industry for targeted analysis as well as compound identification. An overview of applications of LC-MS to wine analysis was presented in Chapter 3, highlighting the scope and utility of the technique in this field. Chapter 4 provided an overview of wine analysis from an African perspective, which clearly illustrates the limited application of the technique on the continent at present.

This dissertation focused specifically on the application of tandem quadrupole LC-MS (LC-qMS/MS) for targeted analysis as well as compound identification in wine analysis. In the experimental sections, several applications of LC-qMS/MS for wine analysis are demonstrated. These may broadly be grouped into two distinct types of analysis. Firstly, qMS/MS was used in multiple reaction monitoring (MRM) mode for sensitive and selective targeted determination of natamycin (Chapter 5), ethyl carbamate (Chapter 6) and 3-alkyl-2-methoxypyrazines (Chapter 7) in wine.

Natamycin is an antimicrobial preservative that is not permitted in wine in the European Union. Suitable analytical techniques are therefore required to regulate the export of South African

wines. In Chapter 5, the development of a rapid and sensitive LC-qMS/MS method for the determination of natamycin in wine was described. Furthermore, kinetic parameters for the degradation of natamycin in wine were reported for the first time.

Ethyl carbamate is a natural carcinogen that occurs at trace level amounts in alcoholic products. A novel method for its determination was reported in Chapter 6. The LC-qMS/MS method is simple and robust and has sufficient sensitivity for its quantitation at natural levels of occurrence in South African wines and spirits.

3-Alkyl-2-methoxypyrazines are ultra-trace-level aroma compounds that contribute to the varietal character of Sauvignon blanc wines. Their analytical determination is difficult due to their low concentrations (in the nanogram per litre range). The method reported here exploits the inherent loadability of LC and the sensitivity and selectivity of qMS/MS to achieve very low limits of detection. Four 3-alkyl-2-methoxypyrazines were quantified in South African Sauvignon blanc wines, including the first quantitative analysis of 3-ethyl-2-methoxypyrazine.

Finally, in Chapter 8, the neutral loss and survey scan modes of LC-qMS/MS were utilised for the improved analysis of the complex red wine anthocyanin fractions. By exploiting the predictable elimination of the sugar moiety in neutral loss mode, highly selective detection of glycosylated anthocyanins and derivatives was achieved. Concurrent survey scan experiments were used to unambiguously identify neutral loss detected compounds.

9.2. Conclusions

The goal of this study was the detailed evaluation of LC-qMS/MS for application to wine analysis. The results presented clearly demonstrate the utility of the technique for solving analytical problems relevant to the wine industry. For targeted analysis in MRM mode, the technique contributes unrivalled sensitivity to applications involving liquid phase separations. In addition, this mode offers sufficient specificity to identify targeted compound with a high degree of certainty. These attributes make LC-qMS/MS ideally suited for accurate trace level quantitation of targeted analytes in wine with a high degree of qualitative certainty, adequately demonstrated for natamycin, ethyl carbamate and methoxypyrazines. The benefit of this approach is clear from the data. LC-qMS/MS is now routinely used for regulation of natamycin concentrations in South African export wines, while the data obtained for methoxypyrazines and ethyl carbamate represent the most comprehensive data for these compounds in South African

wines reported to date. The developed methods are currently used to regulate this industry in terms of the food safety and quality assurance mandate of the Department of Agriculture, Forestry and Fisheries.

Furthermore, qMS/MS also offers several scanning modes suitable for structure elucidation and the identification of unknown wine compounds. The advantages of LC-qMS/MS for compound identification were clearly demonstrated for red wine anthocyanin analysis. The primary benefits of this approach are two-fold. Firstly, increased selectivity simplifies identification and minimises co-elution so that the structured elution may be exploited to allow more accurate tentative compound identification. Secondly, product ion spectra significantly increase the certainty in peak identification, which is an important factor due to the unavailability of standards for wine anthocyanins. The method therefore offers a simple and fast approach for qualitative screening of red wine anthocyanins where the use of LC separation overcomes some of the limitations of direct infusion MS such as ion suppression as well as wrong assignment of compounds due to identical masses of many wine pigments.

9.3. Perspectives

Several important considerations concerning the application of LC-qMS/MS to wine analysis were highlighted in the work reported in this dissertation. First of all, the complexity of the wine matrix dictated the development of dedicated and specialised sample preparation protocols for each application. Despite the advantages of LC-qMS/MS, sample preparation remains an essential step in the analysis of complex samples such as wine, and as such, represents an essential part of method development.

Related to this is the fact that LC-MS is challenged by analyte ionisation; atmospheric pressure ionisation sources may fail to ionise some analytes. While atmospheric pressure chemical ionisation is mostly free from matrix interference effects, electrospray ionisation is affected by this phenomenon. Therefore various ionisation sources may be required to fully exploit the benefits of LC-MS, whereas effective sample preparation can be used to overcome some of these limitations.

Although not addressed in this study, MS lends itself to the use of isotopically labelled internal standards, which can be used effectively to overcome matrix interference effects for quantitative

purposes. Despite the costs involved in the use of isotopically labelled standards, their use may further improve quantitative accuracy.

Finally, it should be noted that several alternative MS architectures, such as ion-trap, time-of-flight and Fourier transform ion cyclotron analysers, offer specific advantages compared to qMS/MS instruments evaluated in this work. High resolution instruments are capable of accurate mass determination and therefore offer an alternative strategy towards compound identification and structure elucidation. However, these instruments are more expensive and currently less readily available in the wine industry. The prohibitive capital layout required for these instruments currently limits their application in wine analysis, but their distinct advantages should ensure many future applications to this field.