DEVELOPMENT OF A NOVEL LC-MS/MS METHOD FOR THE DETECTION OF ADULTERATION OF SOUTH AFRICAN SAUVIGNON BLANC WINES WITH 3-ALKYL-2-METHOXYPYRAZINES.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis 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.

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Summary

A method for the detection of adulteration of South African Sauvignon blanc wines, by enrichment with foreign sources of 3-alkyl-2-methoxypyrazenes, is described. The levels of three 3-alkyl-2-methoxypyrazenes (3-isobutyl-, 3-isopropyl- and 3-*sec*-butyl-2-methoxypyrazine) in South African Sauvignon blanc wines were measured with liquid chromatography-mass spectrometry. Sample preparation involved clean-up and pre-concentration by distillation followed by solvent extraction of the distillate with dichloromethane. Extracts were acidified and concentrated by evaporation and finally reconstituted to a fixed volume to affect quantitative pre-concentration of the samples. Sample extracts were separated with reversed phase liquid chromatography utilizing a phenyl-hexyl separation column. Residues were measured with liquid chromatography-mass spectrometry utilizing a tandem quadrupole mass spectrometric detector operated in multiple reaction monitoring mode for optimal trace level quantitation. Atmospheric pressure chemical ionization was utilized as electrospray ionization was found to suffer from quenching effects attributed to the sample matrix. Qualitative information was obtained from the relevant molecular ions as well as two secondary ion transitions (and one ion ratio) in each case. Recoveries obtained by the extraction procedure were better than 90% with coefficient of variance of better than 10% at concentrations from 1 to 100 ng/L. The limit of detection of the method was 0.03 ng/L and the limit of quantification 0.10 ng/L for the three analytes measured. The described LC-MS method is more sensitive for the determination of 3-alkyl-2-methoxypyrazines in wine than GC methods reported for the same purpose.

From the experimental data, a set of parameters were established to discriminate adulterated South African Sauvignon blanc wines. It was demonstrated that the 3-isobutyl-2-methoxypyrazine concentration, despite showing considerable variance, was confined to a relatively narrow range spanning approximately two orders of magnitude (0.20 to 22 ng/L). A clear indication of possible maximum

values for this compound in South African Sauvignon blanc wines was obtained from the analysis of a large number of samples (577), spanning most relevant wine producing regions and representing vintages 2003 to 2006. It was also demonstrated that South African Sauvignon blanc wines contain the major 3-alkyl-2-methoxypyrazenes in reasonably distinct relative amounts and that the said ratios of abundance may be used to elucidate authenticity. The expected effect of adulteration with green pepper extracts or some synthetic preparations on the 3-isobutyl-2-methoxypyrazine concentration as well as the relative abundances were also determined by characterizing the corresponding profiles in green peppers and some synthetic flavor preparations. Two adulterated samples in the dataset were identified by both outlined criteria. A limited number of wines of other cultivars were also analyzed. The results represent the most complete and accurate data on the 3-alkyl-2-methoxypyrazine content of South African Sauvignon blanc wines to date.

A publication covering the work presented in this thesis is currently in preparation.

Opsomming

`n Metode word beskryf vir die opsporing van vervalsing van Suid-Afrikaanse Sauvignon blanc wyn met wynvreemde bronne van 3-alkiel-2-metoksiepirasiene om die soetrissiegeur daarvan te bevorder. Die vlakke van drie 3-alkiel-2-metoksiepirasiene (3-isopropiel-, 3-isobutiel- en 3-*sek*-butiel-2-metoksiepirasien) is bepaal deur middel van vloeistofchromatografie-massaspektrometrie. Monstervoorbereiding behels distillasie gevolg deur vloeistofekstraksie met dichlorometaan. Ekstrakte is aangesuur en die oplosmiddel is afgedamp waarna dit opgemaak is tot ’n bekende volume. Die ekstrakte is met feniel-heksiel gebaseerde omgekeerdefase vloeistofchromatografie geskei en die vlakke van drie 3-alkiel-2-metoksiepirasiene in Suid-Afrikaanse Sauvignon blanc wyn is met behulp van massaspektrometrie gemeet. Die massaspektrometer is in multi-reaksie moniteringsmodus gebruik om optimale spoor-vlak kwantifisering te bewerkstellig terwyl kwalitatiewe inligting uit ioon-oorgange en verhoudings verkry is. Positiewelading atmosferiesedruk chemiese ionisasie is gebruik nadat bevind is dat elektrosproei-ionisasie deur die matriks onderdruk is. Die herwinning met die metode behaal, is beter as 90% met koeffisiënt van variasie van beter as 10% by konsentrasies tussen 1 en 100 ng/L. Die deteksielimiet was 0.03 ng/L en kwantifiseringslimiet 0.10 ng/L, vir al drie analiete. Die metode bied beter sensitiwiteit vir die bepaling van 3-alkiel-2-metoksiepirasiene in wyn as gaschromatografiese metodes wat vir dieselfde doel aangewend is.

Die eksperimentele data is gebruik om parameters vas te stel waarvolgens vervalsde wyn onderskei kon word. Dit is gedemonstreer dat die 3-isobutiel-2-metoksiepirasienkonsentrasie, ten spyte van beduidende variasie, beperk is tot ’n redelike nou konsentrasiegebied. Die vlakke het gevarieer oor twee ordegroottes, tussen ongeveer 0.20 en 22 ng/L. Hierdie inligting het egter ’n duidelike aanduiding van moontlike perke wat vir die komponent in Suid-Afrikaanse Sauvignon blanc wyn verwag kan word, gelewer aangesien ’n statisties beduidende aantal monsters ontleed is (577) en die relevante produserende

areas goed verteenwoordig is. Oesjare tussen 2003 en 2006 is ook goed verteenwoordig in die studie. Verder is dit ook duidelik dat Suid-Afrikaanse Sauvignon blanc wyn die drie vernaamste 3-alkiel-2-metoksiepirasiene in redelike konstante relatiewe hoeveelhede bevat. Die relatiewe verhouding van die genoemde stowwe is ook in soetrissie en sintetiese middels bepaal en aangesien die verhouding daarvan in wyn beduidend verskil het van die in soetrissie en sintetiese middels, kon vervalsing ook hieruit bepaal word. Die verwagte invloed van vervalsing met soetrissie en sintetiese middels op die relatiewe hoeveelhede in wyn kon gevolglik bepaal word. Hierdie twee faktore kon dus gebruik word om vervalsing van Suid-Afrikaanse Sauvignon blanc wyn met soetrissie ekstrakte en sintetiese middels te bepaal. Twee vervalsde wyne is met beide strategieë gëidentifiseer. `n Beperkte aantal wyne van ander kultivars is ook met die metode ontleed. Die resultate wat in hierdie studie verkry is, verteenwoordig die volledigste inligting betreffende die 3-alkiel-2-metoksiepirasiene in Suid-Afrikaanse Sauvignon blanc wyn.

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Abbreviations

APCI	atmospheric pressure chemical ionization
cGC	capillary gas chromatography
CI	confidence interval
CL	confidence limits
DAD	diode array detector
DC	direct current
DCM	dichloromethane
DVB	divinylbenzene
EI	electron-impact ionization
EMP	3-ethyl-2-methoxypyrazine
ESI	electrospray ionization
EU	European Union
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
HPLC	high-performance liquid chromatography
IBMP	3-isobutyl-2-methoxypyrazine
ID	internal diameter
IPEP	3-isopropyl-2-ethoxypyrazine
IPMP	3-isopropyl-2-methoxypyrazine
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
m/v	mass per volume
m/z	mass to charge ratio
MDL	minimum detection limit
MMP	3-methyl-2-methoxypyrazine
MQL	minimum quantification limit
MRM	multiple reaction monitoring
MS	mass spectrometer
ND	not detected
NPD	nitrogen-phosphorus detector
NQ	not quantified
PC	principal component
PCA	principal component analysis
PDMS	polydimethylsiloxane
PEG	polyethylene glycol
PSDVB	polystyrene divinylbenzene
Q	quadrupole analyzer
QTOF	quadrupole time-of-flight
RF	radio frequency
RP-LC	reversed phase liquid chromatography
RSD	relative standard deviation
S/N	signal-to-noise ratio
SBMP	3-*sec*-butyl-2-methoxypyrazine
SBSE	stir bar sorptive extraction
SDB	styrene-divinylbenzene
SIM	single-ion monitoring
SPE	solid phase extraction
SPME	solid phase microextraction
Stdev	Standard deviation
TIC	total ion chromatogram
TOF	time-of-flight
UV	ultraviolet
UV-Vis	ultraviolet visible
v / v	volume-per-volume

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CHAPTER 1

Introduction

1.1. Historical perspective on wine and adulteration

Wine may have an archeological origin dating back more than 7.5 thousand years, with the earliest suspected wine residues dating from the early to mid-fifth millennium B.C. Clear evidence of intentional winemaking first appears in representations of wine presses that date back to the reign of Udimu in Egypt, some 5000 years ago. The development of wine-making and the domestication of the wine grape, *Vitis vinifera*, are thought to have occurred in southern Caucasia, an area that includes parts of present-day north-western Turkey, northern Iraq, Azerbaijan and Georgia. Domestication may also have occurred independently in Spain.

The evolution of wine-making from an infrequent occurrence to a routine agricultural event may have followed the development of a settled agricultural lifestyle. At the same time, beneficial properties such as low mineral and water requirements as well as excellent regenerative powers and woody structure, which have permitted the grapevine to withstand considerable winterkill while producing acceptable yields in cool climates, favored its cultivation and the spread of viticulture. For ancient humans, the result of grape fermentation was the transformation of a perishable, periodically available fruit into a relatively stable beverage with novel and potentially intoxicating properties. Wine also developed an association with religious rites with much symbolic value.

From Caucasia, grape growing and wine making spread into Palestine, Syria, Egypt and Mesopotamia and from this base wine consumption and its socio-religious connections reached the Mediterranean. In more recent times, European exploration and colonization have spread grapevine cultivation into most of the temperate climatic regions of the globe. Wines began to take on

their modern expression in the 17th century when the use of sulfur in barrel treatment are thought to have become widespread, thus greatly increasing the likelihood of producing better-quality wines and extending their aging potential. The utilization of glass bottles and cork as a closure in the 17th century provided conditions favorable for the production of modern wine. With the discovery by Pasteur around 1860 of the central importance of yeasts and bacteria to fermentation, the chain of events was set in motion that has produced the incredible range of wines that typifies modern commerce. From these humble origins, grape production has developed into the world's most important fresh fruit crop. Worldwide grape production in 1992 exceeded that for oranges, bananas or apples. The area planted under grapevines in 1990 was estimated at about 8.7 million hectares of which approximately 71% of the yield was fermented into wine.[1]

South Africa has a long history of winemaking, dating back to 1655, when Jan van Riebeeck planted the first vines in the Cape. In 1659 the first wine was made from these plantings. In the late 18th and early 19th centuries the Cape wine industry became famous for Constantia, a sweet, fortified wine that was much sought-after in the royal courts of Europe and was venerated by writers of that time. Napoleon Bonaparte reputedly requested a bottle of Constantia on his deathbed. The specialization in fortified wine production is still evident in the South African wine industry today as about half of its capacity is dedicated to fortified wine and brandy. In modern times the South African wine industry started to blossom after the Second World War, with the perfection of cold fermentation techniques for white wines. Since the recent transition to a democracy, South African wine exports proliferated, mainly to the United Kingdom, the Netherlands and other European destinations. The explosive growth in wine exports from South Africa are demonstrated by the fact that it increased from 855 000 cases in 1990 to 15.4 million cases in 2000, an incredible 1 700 percent increase. At the present day, the South African industry is changing from predominantly white to red wines, as is reflected by the fact that 75% of new plantings are red varieties, particularly Cabernet Sauvignon and Shiraz. This transition reflects the fact that the climate in South Africa is more conducive to Bordeaux and Rhône-style wines.[1,2] South Africa

also posses a red variety of own, Pinotage, which is a cross between Pinot Noir and Cinsaut.[3]

The thorny issue of defining exactly what is wine has historically been contentious and remains difficult to answer to this date. The variability and value of wine have traditionally made it a target for unscrupulous operators and the wine trade has been beset with adulteration and fraud throughout its history. The long human chain stretching from grower to consumer affords many opportunities for illegal practices. As early as the first century A.D., Pliny the Elder bemoaned the fact that not even the nobility was exempt from falling victim to fraudulent practices in the wine trade of his time.

It is also important to recognize that the law viewed the same procedures differently through the ages, sometimes condoning and sometimes condemning identical practices. The simplest and most obvious form of adulteration is the addition of water to the product to increase the volume. Dilution of wine with water was however an accepted practice in ancient Greece. Another obvious means of increasing volumes of wine is to blend it with spirits or other inferior wines. This practice was common among Bordeaux merchants of the 18th century who blended fine clarets destined for the English market with rough wines imported from Spain, the Rhône or the Midi, to increase profits. Systematization by the Portuguese government of the practice of adding Brandy to wine eventually led to the production of Port as it is known today.[4] One particularly controversial method of altering the nature of wine is the addition of sugar during fermentation to increase the eventual alcoholic strength, a practice also known as Chaptalization.

One of the most common forms of fraud does not involve the addition of any substance to the product, but merely the label. An early example of fraud involving the region of origin of wine from Roman times, involved passing ordinary wines off as valuable Falernian, the most highly prized Italian wine from that period. Today, controlled appellations, a method based on the French system, are widely used for labeling wine and designating quality and geographical delimitation. The adoption of controlled appellation systems as

well as regulations and legislation served to create the legal apparatus to combat fraud and adulteration so that although once rife, it is considerably rarer in the wine trade of today.[4,5]

1.2. Sauvignon blanc wine

Sauvignon blanc is the vine variety solely responsible for some of the world's most popular and most distinctive dry white wines with the best examples of the cultivar produced in France, particularly those from Sancerre and Pouilly-Fumé.[5] Sauvignon blanc is also one of the most important white wine cultivars in South Africa.[6] In 2004, 6944 ha of Sauvignon blanc were cultivated in South Africa, which represented 12.8% of white wine varieties and 6.9% of total wine variety plantings.[7] Sauvignon blanc's most identifiable characteristic is its piercing, instantly recognizable aroma, described as various nuances of green, grassy, herbaceous, green pepper, asparagus and gooseberry.[5,6,8] Variously substituted 3-alkyl-2-methoxypyrazines are known to contribute to the distinctive vegetal and herbaceous character associated with wine of this cultivar.[6,8,9]

Sauvignon blanc wine is extremely sensitive to climatic, viticultural and production factors, partly due to the temperature and light-sensitivity of the 3-alkyl-2-methoxypyrazines.[6,9,10] In South Africa, Sauvignon blanc grapes ripen early in mid-season. At optimum maturity the average sugars are 21 to 24° Balling with a total titratable acidity of 6 to 7 g/L.[11] In the grapes, the concentration of 3-alkyl-2-methoxypyrazines decrease as a result of solar exposure during ripening.[9,10] Consequently, in unsuitable, hotter climates, high concentrations at harvest is usually associated with a lack of ripeness and may have a negative impact on wine aroma quality.[6] Sauvignon blanc wine is therefore better adapted to cooler climates as higher overall aroma concentrations are observed in wines produced in the cooler regions.[6] The South African climate is therefore generally not conducive to the production of Sauvignon blanc wine possessing the desired and characteristic vegetal and herbaceous character associated with products from France and New

Zealand. In South Africa, Sauvignon blanc wines with a pungent and precise varietal character are only produced in cool localities and by applying specific canopy management and enological practices.[9,12] A number of outstanding Sauvignon blanc wines have nevertheless been produced in South Africa, contrary to the climatological suitability of the region and to the surprise of eminent wine-writers.[12]

Flavor and aroma not only contributes to the varietal and regional distinctiveness of wine, but also has a dramatic influence on the price of the product. Adulteration of some South African Sauvignon blanc wines by enrichment with foreign sources of 3-alkyl-2-methoxypyrazines have recently been confirmed.[13] It is suspected that the alkylmethoxypyrazine levels in the adulterated wines were enriched with extracts obtained from green peppers. The possibility that synthetic alkylmethoxypyrazine preparations, which are widely available in the food industry, were also used for the same purpose cannot be excluded.

South African and international standards prescribe that wine shall be the product of fermented grape juice and that wine shall be considered adulterated when a foreign, unapproved substance is added to the product.[14] Premium wine quality should therefore strictly be achieved through a favorable balance between fruit-, fermentation- and processing-derived flavor and aroma. Adulteration of wine may have an adverse effect on the South African wine export industry. Wine exported in 2004 amounted to 269 million liters, which is 39% of the total production of wine for that year.[7] The continued accessibility of wine export markets to South African producers is regarded as of the utmost importance to the economy of the wine producing regions of South Africa. It is therefore imperative that the extent of possible adulteration in the South African Sauvignon blanc industry be investigated.

The enforcement of the laws is a political-economic decision and the effectiveness of regulations depends largely on the willingness and ability of enforcing agencies to assess compliance. However, the technical ability to assess compliance is within the realm of science. The objective of this

dissertation is therefore to establish an unambiguous method for the elucidation of authenticity of South African Sauvignon blanc wine, specifically regarding adulteration with foreign sources of 3-alkyl-2-methoxypyrazines.

1.3. Origin, chemical properties and flavor characteristics of methoxypyrazines

Pyrazines (1,4-diazines) are nitrogen-containing heterocyclic compounds that are widely distributed in nature.[15] The 3-alkyl-2-methoxypyrazines are important flavor components due to their extremely low sensory detection thresholds.[6,15,16,17] Various 3-alkyl-2-methoxypyrazines have been identified in a number of materials of vegetable origin where they contribute significantly to the characteristic aroma of each species.[15,17] Three 3-alkyl-2-methoxypyrazines, namely 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (SBMP) are particularly dominant in several vegetable species.[18] In many cases all three mentioned 3-alkyl-2-methoxypyrazines are present, with one compound often clearly dominant.[17] In green- and red peppers, the isobutyl compound predominates while the *sec*-butyl compound predominates in carrot, parsnip, beetroot and silverbeet and the isopropyl compound in peas, broad beans, cucumber and asparagus.[17] Although possessing higher odor thresholds, 3-ethyl-2-methoxypyrazine (EMP) and 3-methyl-2-methoxypyrazine (MMP) were also identified in some materials of vegetable origin.[10,15,19] Table 1.1. presents a summary of the odor threshold and flavor description of the 3-alkyl-2-methoxypyrazines under investigation.[8,10,15,16,17,20]

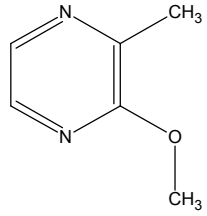
Table 1.1.: Odor threshold and flavor properties of the relevant 3-alkyl-2-methoxypyrazines.[8,10,15,16,17,20]

Compound	Odor threshold in water (ng/L)	Flavor description
IBMP	1 to 2	Bell peppers
IPMP	1 to 2	Bell peppers, green peas
SBMP	1 to 2	Galbanum, ivy leaves, green peas
EMP	425	Raw potato
MMP	4 000	Roasted peanuts

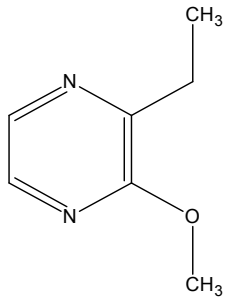
Comprehensive physical and chemical data pertaining to the compounds of interest were not available, Table 1.2. presents some properties of pyrazine (1,4-diazine), a related compound. A boiling point of 50 ºC was however reported for IBMP in a literature reference which may provide an indication of the volatility of these compounds (less substituted congeners expected to be more volatile).[21]

Table 1.2.: Physical and chemical of pyrazine (1,4-diazine).[22,23]

Compound	pK_1 (27°C)	pK_2(27°C)	mp (°C)	bp (°C)	density (g/mL)
Pyrazine	0.65	-5.78	51.0	115	1.0311



3-Methyl-2-methoxypyrazine



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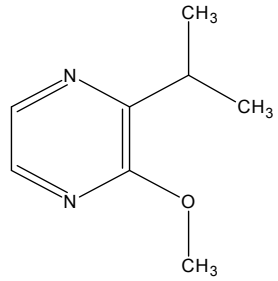
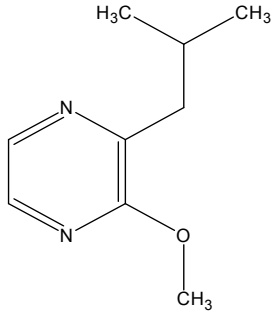
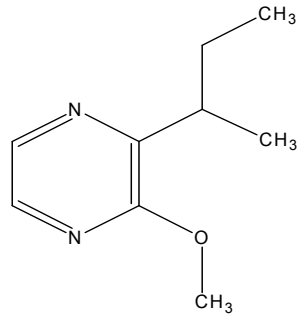


3-Isopropyl-2-methoxypyrazine



3-Isobutyl-2-methoxypyrazine



3-*sec*-Butyl-2-methoxypyrazine

Figure 1.1.: Chemical structure of five 3-alkyl-2-methoxypyrazines relevant to the investigation.

3-Alkyl-2-methoxypyrazines, notably the isobutyl, *sec*-butyl and isopropyl compounds, are also present in several wine grape species including Cabernet Sauvignon grapes (*Vitis vinifera* L. cv. Cabernet Sauvignon) and Sauvignon blanc grapes (*Vitis vinifera* L. cv. Sauvignon blanc).[10,19] 3-Alkyl-2-methoxypyrazines are known to contribute to the typical green pepper, herbaceous character associated with Sauvignon blanc wine.[6,8,10,16] The most abundant congener of the 3-alkyl-2-methoxypyrazines found in Sauvignon blanc wine is IBMP, representing approximately 80% of 3-alkyl-2-

methoxypyrazines commonly found in wine.[6,10,19] Studies also demonstrated that IBMP is the main contributor to the vegetal aroma in Sauvignon blanc wine.[6,8] The fact that IBMP is also the dominant congener in green peppers, may explain the supposed use thereof in the adulteration of wine. The other major 3-alkyl-2-methoxypyrazines commonly found in wine, SBMP and IPMP, each represent approximately 10% of the total 3-alkyl-2-methoxypyrazines in wine.[10,19] The occurrence of EMP in Sauvignon blanc have also been tentatively reported while the possible occurrence of MMP has been suggested based upon a feasible biosynthetic route.[10] Although it is unlikely that EMP and MMP may make a significant contribution to Sauvignon blanc aroma, due to their high odor detection thresholds, they were nevertheless tentatively included in the investigation. If present at all, the levels of EMP and MMP may possibly be attenuated by adulteration of Sauvignon blanc wine with fruit extracts.

The olfactory threshold at which these compounds are sensed is extraordinarily low, values ranging from 0.5 to 2 ng/L in water are reported in the literature for the alkylmethoxypyrazines.[16,19,20,24] The detection threshold of IBMP in red wine is 10 to 15 ng/L while 1 to 2 ng/L has a significant influence on the aroma of a methoxypyrazine-free white wine.[8,19,20,24] The ability of a specific compound to impact the aroma of a wine depends on the specificity of the aromatic note of such a compound.[25] In addition to the fact that the vegetable character of wine may primarily be attributed to the presence of IBMP, Ferreira *et al.* reported that other volatile wine aroma compounds may synergistically interact with IBMP to significantly enhance the perceived pepper odor nuance.[26] On the contrary, components such as fusel alcohols, acids, esters, ß-damascenone and some volatile phenols are not able to individually affect the aroma of wine even if they are present at concentrations well above their odor thresholds.[25] It may therefore be concluded that IBMP, present at levels of the order of a few parts per trillion, may have a significant impact on the aroma of Sauvignon blanc wine.

Methoxypyrazine concentrations in grapes are influenced by a multiplicity of factors including grape variety, fruit maturity, season, climate and solar

exposure of the fruit.[6,9,19] South African Sauvignon blanc wines contain approximately < 1 to 14 ng/L of IBMP and often possess very little or no cultivar character as far as the typical grassy, green pepper aroma is concerned.[6] As the minor 3-alkyl-2-methoxypyrazines are expected to be present at levels of no more than approximately 10% of that of IBMP, these may occur at levels of the order of < 0,1 to 2 ng/L respectively, but no relevant quantitative data are currently available.[19] Australian Sauvignon blanc wine, produced under similar climatological conditions, contains approximately 2 to 15 ng/L of IBMP.[6] France and New Zealand, which have cooler climates and are famous for producing high quality Sauvignon blanc wines, typically range from 5 to 40 ng/L and 10 to 35 ng/L IBMP respectively.[6,12]

1.4. Analytical methodologies for the analysis of methoxypyrazines in wine

Several analytical methodologies have been employed successfully for the measurement of selected 3-alkyl-2-methoxypyrazines in wine. These exclusively comprise gas chromatography, either in conjunction with mass spectrometric detection [10,18,24,27,28,29] or nitrogen-phosphorous selective detection (NPD).[20,27] Due to the very low levels of the analytes, sample pre-concentration is indispensable in all methods used. Sample preparation generally involves clean-up and pre-concentration utilizing various techniques including liquid-liquid extraction [10,18,24,28,29,30] distillation [10,18,20,28,29], solid phase extraction [10,18,28,29] and ionic strength adjustment for the headspace techniques.[20,27] Sample introduction commonly entails splitless injection of concentrated extracts [10,18,24,29,30] or headspace solid phase micro-extraction (SPME) utilizing various fibers.[20,27] An internal standard is universally used in conjunction with the various gas chromatographic techniques while the choice of internal standard varied between deuterium labeled analogs [10,18,27,28,30] and differently substituted pyrazines.[20,24,29] Table 1.3. presents a concise summary of some methods of analysis reported for the determination of various 3-alkyl-2-methoxypyrazines in wine.

Table 1.3.: Summary of methods reported in the literature for the analysis of methoxypyrazines in wine.

Author	Analysis technique	Sample preparation and introduction	Detection	Analytes	Performance of the method
Kotseridis *et al.*[24]	Capillary gas chromatography (cGC) utilizing Carbowax-20M phase and MMP as internal standard.	Solvent extraction with diethyl ether-hexane (1/1, v/v). Splitless injection of concentrated sample extract.	Mass spectrometer operating in selected ion monitoring (SIM) mode.	IBMP	MQL:[b] 2 ng/L
Sala *et al.*[20]	cGC utilizing two phases for compound identification, CP-Wax and SPB-35. IPEP[a] internal standard.	Headspace solid phase micro-extraction (SPME) utilizing a 65 µm PDMS/DVB fiber. Samples were acidified and distilled followed by neutralization and ionic strength adjustment prior to SPME sampling.	Nitrogen Phosphorous Detector (NPD)	IBMP, SBMP, IPMP, EMP	MDL:[c] 0.3 ng/L (IBMP, IPMP and SBMP). 1.0 ng/L (EMP), (S/N = 3:1)
Roujou de Boubee *et al.*[18]	cGC utilizing a BP20 phase and deuterium labeled IBMP as internal standard.	Steam distillation of samples after pH adjustment with NaOH, extraction of distillate with cation exchange resin, elution with 10% NaOH and finally extraction of aqueous phase with dichloromethane. Splitless injection of concentrated sample extract.	Chemical ionization mass spectrometry. Scan mode (m/z 165 - 172)	IBMP	Calibration started at 2 ng/L.
Ryan *et al.*[27]	Two-dimensional gas chromatography utilizing BPX5 and BP20 phases. d_3-IBMP utilized as internal standard.	Headspace SPME utilizing a 65 µm PDMS/DVB fiber and ionic strength adjustment of samples.	Time-of-flight mass spectrometry (utilizing d_3-IBMP internal standard) and NPD	IBMP, SBMP	MDL(TOF): 1.96 ng/L MDL(NPD): 0.5 ng/L (IBMP only)
Kotseridis *et al.*[30]	cGC utilizing Carbowax-20M phase and deuterium	Solvent extraction with diethyl ether-hexane (1/1, v/v). Splitless	Mass spectrometer operating in SIM	IBMP	MQL: 2 ng/L (S/N = 3:1)

	labeled IBMP as internal standard.	injection of concentrated sample extract.	mode.		
Lacey *et al.*[10]	cGC utilizing deuterium labeled IBMP and IPMP as internal standards.	Distillation of samples followed by extraction of the distillate with cation exchange resin. Resin washed with 15% NaOH and finally extraction from aqueous phase with dichloromethane. Splitless injection of concentrated sample extract.	Mass spectrometry.	IBMP IPMP SBMP	MDL: 0.15 ng/L
Allen *et al.*[28]	cGC utilizing BP 5, DB-1, DB-1701 and DB-Wax phases and deuterium labeled IBMP as internal standard.	Distillation of samples (pH 6) followed by extraction of the distillate with cation exchange resin. Resin washed with 10% NaOH and finally extraction of aqueous phase with dichloromethane. Cold on-column injection of concentrated sample extract.	Mass spectrometry (SIM).	IBMP IPMP	Variable, < 1 ng/L
Hashizume *et al.*[29]	cGC utilizing a DB-Wax phase and 2-Methyl-3-*n*-propylpyrazine as internal standard.	Steam distillation of samples (pH 5) followed by extraction of the distillate with ion exchange resin. Resin washed with 20% KOH / 1 M Na_2CO_3, extraction of aqueous phase with dichloromethane. Splitless injection of concentrated sample extract.	Mass spectrometry (SIM).	IBMP IPMP	0.2 ng/kg (grapes)

[a] = 3-Isopropyl-2-ethoxypyrazine [b] = Minimum quantification limit [c] = Minimum detection limit

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1.5. Detection of adulteration: Characterization of the profile of relative abundance of the major 3-alkyl-2-methoxypyrazines in Sauvignon blanc wine

At present, an unambiguous method for the detection of adulteration of South African Sauvignon blanc wine by fraudulent enrichment of the levels of 3-alkyl-2-methoxypyrazines, does not exist. South African regulatory laboratories, tasked with auditing the Sauvignon blanc industry, utilize the method of Kotseridis *et al.*[24] to build a historical database of quantitative data pertaining specifically to 3-isobutyl-2-methoxypyrazine. Possible cases of adulteration are identified by evaluation of the relevant quantitative data for the region and vintage and comparison with the database of expected values. Due to the variable natural abundance of 3-isobutyl-2-methoxypyrazine in South African Sauvignon blanc wine, a strategy of analyzing the IBMP content of must samples prior to alcoholic fermentation and the final product, has recently been employed in an effort to detect possible cases of adulteration. As IBMP can only be produced while the grapes are on the vine, unexplained increases in the quantity of IBMP during the winemaking process may then provide conclusive evidence of adulteration.

The method of Kotseridis *et al.*[24] (MQL = 2 ng/L) is only suitable for the determination of IBMP in South African Sauvignon blanc wines and is not generally applicable for the determination of 3-alkyl-2-methoxypyrazines at their natural levels of occurrence. Insufficient data pertaining to the relative abundance of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine is therefore currently available.

The characteristic cultivar character associated with Sauvignon blanc wine is in part attributed to certain methoxypyrazines, of which IBMP is the most important.[6,8,9] It may be expected that the other 3-alkyl-2-methoxypyrazine congeners that are present in Sauvignon blanc grapes, such as IPMP and SBMP, contribute to the typical green pepper, herbaceous nuances that

distinguishes the variety, as these may be present at levels above their sensory thresholds.[8,10,19] As different grape cultivars have distinctive sensory properties, it may reasonably be assumed that Sauvignon blanc wine contain these congeners in distinct relative amounts, causing the varietal distinction. This assumption is supported by the findings of Lacey *et al.* who reported that that the relative proportions of three methoxypyrazines in Sauvignon blanc wine of Australian, New Zealand and French origin are fairly constant and that the relative abundance of IBMP to IPMP is approximately 7:1.[10] Allen *et al.* similarly found that the typical abundance of SBMP in some red wines are approximately 2% of that of IBMP.[28] Murray *et al.* reported that greenpeppers contains the three relevant methoxypyrazine congeners in fixed relative amounts and that a ratio of approximately 100:1 exists for IBMP to IPMP.[17]

A possible strategy for the detection of adulteration may therefore be to characterize the profile of relative abundances of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine. If the ratios are found to be consistent, then samples from all regions producing Sauvignon blanc wine should be analysed to determine the amount of variance in the typical pattern so that parameters may be established for authentication of South African Sauvignon blanc wine in this regard. Quantitative data for the different congeners may also be used as supplementary information in cases where adulteration is suspected. Green pepper extracts as well as commercial synthetic preparations should also be analysed to determine the expected effect of adulteration on the typical methoxypyrazine profile.

The investigation may be complicated by the fact that wine, labelled as a single cultivar product, may contain small amounts of wine from a different cultivar. Current South African legislation, effective from January 2006, stipulates that at least 85% of the contents of a single cultivar product shall be derived from grapes of that cultivar. Legislation effective up to December 2005, required that 75% of the contents of a single cultivar wine be derived from grapes of that

cultivar.[14] This implies that, depending on the vintage, single cultivar products may contain up to 25% of wine from a different cultivar. The fact that Sauvignon blanc wines may contain undisclosed amounts of wine from other cultivars, may affect the amount of variance in the relative abundances of the 3-alkyl-2-methoxypyrazines, if a pattern is found to exist.

The timing of the harvest is of critical importance from a viticultural point of view as this determines the properties of the fruit, such as the balance between the natural accumulated sugars and acids, which sets the limit on the potential quality of the wine.[1,5] Some producers of Sauvignon blanc wine in South Africa follow a practice of selective and repeated harvesting to obtain fruit possessing distinct qualities. In the warmer wine-producing regions of South Africa, the IBMP content of Sauvignon blanc grapes decrease during ripening.[9] A proportion of the fruit may therefore be harvested before it has reached optimal maturity to ensure high levels of IBMP. A varietal-typical product may thus be obtained by combining the grapes, which were harvested at different times. This practice may clearly affect the levels of the 3-alkyl-2-methoxypyrazines in the product and possibly their relative abundances.

The overall objectives of the study are therefore to establish a method for elucidation of authenticity of South African Sauvignon blanc wine, specifically regarding enrichment with foreign 3-alkyl-2-methoxypyrazines, and to perform an audit of the industry in this regard. Within this context, the following goals were identified:

1. The implementation of an analytical procedure for the quantitation of various 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wines. This procedure should be capable of accurate and precise measurements in a concentration range of approximately 0.1 to 50 ng/L.

2. Analysis of Sauvignon blanc wine samples representative of the South African industry to determine the absolute levels of 3-alkyl-2-methoxypyrazines and possible ratios of relative abundance that may exist between the various congeners as well as the amount of variance across the typical spectrum of wines. The expected effect of adulteration with green pepper extracts and synthetic green pepper flavor preparations, on the typical spectrum, should also be determined by characterization of the relative abundance in the mentioned substances.

3. Use of this information to establish parameters that may be used to discriminate adulterated wine and to predict the effect of adulteration with green peppers and synthetic green pepper flavor preparations, on the relative abundance of various 3-alkyl-2-methoxypyrazines in Sauvignon blanc wine.

1.6. Proposed methodology for characterizing the profile of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine

A major factor that challenges the investigation of wine flavor and aroma are the extremely low concentrations of the wine flavor components. The concentration of the various 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine are expected to be in the range of approximately < 0,1 to 14 ng/L.[6] Such low levels necessitate highly sensitive and selective extraction and analysis methods for quantitative purposes. Chemical changes that occur during aging are not expected to complicate the investigation. De Boubee *et al.* reported that the IBMP content of one Cabernet Sauvignon and one Sauvignon blanc wine remained unchanged during bottle ageing for three years in a dark cellar.[21]

Due to the very low and variable concentrations expected for the 3-alkyl-2-methoxypyrazines in wine, an efficient sample clean-up and pre-concentration

step is essential for the successful measurement of the substances under investigation. The sample preparation procedure should offer quantitative pre-concentration of the analyte and be robust in order to be suitable for the analysis of large numbers of samples. Various sample preparation techniques were evaluated for this purpose including solvent extraction, distillation, solid phase extraction (SPE), and stir bar sorptive extraction (SBSE). Solvent extraction, in combination with distillation, was finally selected as the sample preparation technique as it is particularly suitable for the isolation and pre-concentration of trace quantities of a species.[31]

Liquid chromatography-mass spectrometry (LC-MS) was selected as the analytical technique for measuring the levels of 3-alkyl-2-methoxypyrazines despite the fact that practically all relevant publications report the use of gas chromatography for this purpose. The LC-MS mass selective detector offers sensitivity and selectivity of the same order as equivalent gas chromatography mass spectrometry (GC-MS) detectors. The liquid chromatographic technique however offers advantages over gas chromatography such as higher sample loading capacity and superior sample introduction precision. The latter obviates the requirement for an internal standard for quantitation as is the case with gas chromatography. Liquid chromatography is also better suited for the analysis of thermally unstable components and can accommodate acids and non-volatile solvents, which may be indispensable as part of an efficient sample preparation procedure. Very high electrospray ionization efficiencies, up to 100%, have recently been reported for LC-MS. The compounds of interest are also aromatic, incorporating heteroatoms with lone pairs of electrons in their ring structure, and contain electron donating methoxy groups and alkyl side-chains, structural characteristics that aid charge stabilization in positive mode ionization. These factors suggest that the LC-MS technique may provide very efficient and sensitive detection of the compounds of interest.[32] The advantages associated with the liquid chromatographic technique are therefore expected to outweigh the superior resolution attainable with gas chromatography so that better overall

sensitivity is expected from a LC-MS method. The lower resolving power of liquid chromatography compared to gas chromatography, may however place particularly stringent demands on sample clean-up, separation and mass selective detection processes.

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CHAPTER 2

Analytical techniques

2.1. Introduction

Generally, methods for chemical analysis are at best selective, few are truly specific. Consequently, separation of analytes from potential interferences is vitally important in analytical investigations.[1] This requirement becomes even more important in trace level analysis where the concentration of the analyte, relative to sample matrix components, may be exceedingly small. Modern analytical separations are most commonly performed using chromatography and electrophoresis. Especially the modern versions of high performance liquid chromatography (HPLC) and capillary gas chromatography (cGC) are by far the most widely used separation techniques.[1,2,3]

In this Chapter an overview of the analytical techniques relevant to the study are presented. Apart from a broad introduction to chromatography, the focus of the discussion will primarily be on liquid chromatography and more specifically on the techniques used for analyses of residues in this study. A brief overview of gas chromatographic techniques, relevant to the study, will also be presented.

2.2. General description of chromatography

In chromatographic separations the sample or analyte molecules are transported in a mobile phase through an immiscible stationary phase, which is fixed in a column or on a solid surface. The sample is dissolved in the mobile phase, which may be a gas, a liquid or a supercritical fluid. The two phases are selected to ensure that components of the sample distribute themselves between the mobile

phase and the stationary phases to varying degrees. With the flow of the mobile phase, those components that are retained weakly by the stationary phase elute from the system before strongly retained components. As a consequence of these differences in mobility, components may be separated into discrete bands that can be analyzed quantitatively and/or qualitatively.[1,2,3,4,5]

Chromatographic methods are categorized based upon the physical means by which the mobile and stationary phases are brought together. In column chromatography, the stationary phase is held in a tube, generally referred to as a column, through which the mobile phase is forced under pressure or gravity. In planar chromatography, the stationary phase is supported on a flat surface through which the mobile phase moves by capillary action or under the influence of gravity. Separation in both planar and column chromatography are based upon the same chemical equilibria.[1,2,3,4,5] A more fundamental classification of column chromatographic methods is based on the types of mobile and stationary phases and the equilibria involved in the transfer of solutes between the phases, as is shown in Table 2.1.

Table 2.1.: Classification of the most common column chromatographic separations.[1]

Classification	Method	Stationary phase	Equilibrium
Liquid chromatography	Liquid-liquid or partitioning	Liquid adsorbed on solid	Partition between immiscible liquids
	Liquid-bonded phase	Organic species bonded to solid	Partition between liquid and bonded phase
	Liquid-solid or adsorption	Solid	Adsorption
	Ion exchange	Ion exchange resin	Ion exchange
	Size exclusion	Liquid in interstices of polymeric solid	Partition/sieving
Gas chromatography	Gas-liquid	Liquid adsorbed on solid	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to solid	Partition between gas and bonded phase
	Gas-solid	Solid	Adsorption
Supercritical-fluid chromatography	Supercritical fluid-bonded phase	Organic species bonded to solid	Partition between super-critical fluid and bonded surface
	Supercritical fluid-solid	Solid	Adsorption

2.2.1. Migration rates of solutes in chromatographic separations

Chromatographic separation depends on the relative rates at which different solutes move down the column. These rates are determined by the equilibrium constants for the reactions by which the solutes distribute themselves between the mobile and stationary phases. The distribution constant, K is described by the equation:

$$K = c_S/c_M \qquad (1)$$

where c_S and c_M is the molar concentration of the solute in the mobile and stationary phases, respectively. Ideally, K is constant over a wide range of solute concentrations which results in characteristics such as symmetric Gaussian type peaks and retention times that are independent of solute concentration.[1]

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, while retention time represents the total time that the solute spends in the column.

The retention factor, k' for solute A is defined as follows:

$$k'_A = (K_A V_S)/V_M = (t_R - t_M)/t_M \qquad (2)$$

where K_A is the distribution constant, V_S and V_M are the phase volume of the stationary and mobile phases and t_R and t_M the retention time of the solute and an unretained peak, respectively. Ideally, separations are performed under conditions in which the retention factor for the solutes is in the range 2 to 10.[1]

The selectivity factor, α, of a separation for two species A and B is defined as follows:

$$\alpha = K_B/K_A = ((t_R)_B - t_M)/ ((t_R)_A - t_M) \qquad (3)$$

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 time of the solute and an unretained peak respectively.[1]

The resolution, R_S of a column provides a quantitative measure of its ability to resolve two solutes, A and B, in a mixture:

$$R_S = 2[(t_R)_B - (t_R)_A] / (W_A + W_B) = 2 \Delta t_R / (W_A + W_B) \qquad (4)$$

where $(t_R)_B$ and $(t_R)_A$ are the retention time of solute A and B and W_A and W_B, the width of the peaks at the baseline (Figure 2.1.). A resolution of 1.5 provides complete baseline separation of two components.[1,2,3]

As the peak widths of two adjacent peaks in high efficiency chromatography are approximately equal, the resolution equation may also be written in terms of α, k' and N:

$$R_S = ((N)^{1/2}) / 4 \,.\, ((\alpha - 1) / \alpha) \,.\, (k_2 / 1 + k_2) \qquad (5)$$

where N is the plate count, α the selectivity factor and k_2 the retention factor of the last eluting solute.[2,4]

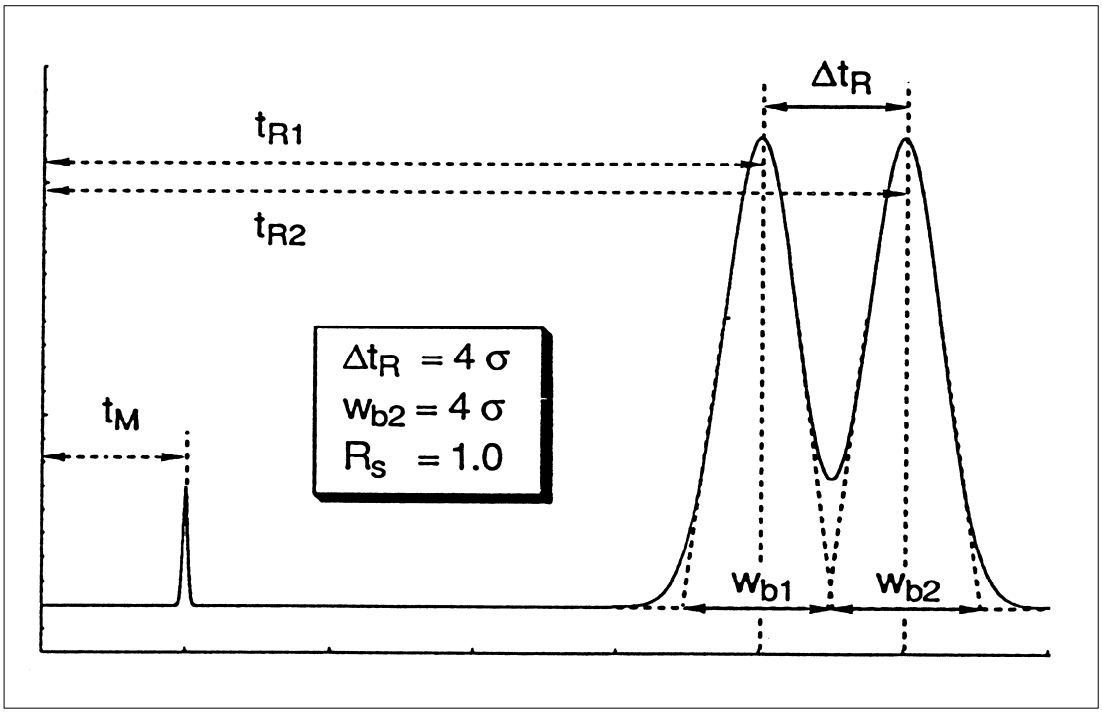

Figure 2.1.: Resolution for Δ t_R = 4 σ.*

* Introduction to Separation Science, Stellenbosch University 2007, P. Sandra, A.J. de Villiers.

2.2.2. Band broadening in chromatography

In any chromatographic process, separation is generally accompanied by dilution of the analyte, a phenomenon commonly referred to as band or peak broadening.

Peak broadening predominantly occurs as the sample is separated on the column, but may also occur outside the column. Extra-column band broadening includes the dilution attributed to the injector, connecting tubing as well as the detector.[1] The discussion that follows pertains specifically to on-column peak broadening.

Chromatographic peaks generally resemble Gaussian curves because variable residence time of the solutes in the mobile phase leads to irregular migration rates with a symmetric spread of velocities around the mean value. The breadth of a Gaussian curve is directly related to the variance, σ^2 of measurement. The efficiency of a column is therefore conveniently expressed in terms of variance per unit length. The plate height, H is given by the equation:

$$H = \sigma^2/L \qquad (6)$$

where L is the length of the column and σ^2 carries units of length squared. H therefore represents a linear distance in centimeters. The plate height may be thought of 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.[1,3]

The plate count, N is related to H by the equation:

$$N = L / H \qquad (7)$$

where L is the length of the column packing. N may also be approximated by determining $W_{1/2}$, the width of the peak at half-height. The plate count is then given by:

$$N = 5.54 \, (t_R / W_{1/2})^2 \qquad (8)$$

where t_R is the retention time of the peak. The efficiency of the chromatographic column increase as the plate count becomes greater. The plate count N and plate height H may be used to measure column performance. Where two columns are compared, the same compound should be used in determining these parameters.[1]

Peak broadening during the chromatographic separation is the consequence of longitudinal diffusion, multiple flow paths through a packed bed and the finite rate at which several mass-transfer processes occur. The contribution of each of these processes to the plate height is described by the Van Deemter equation:

$$H = A + B/u + (C)u \qquad (9)$$

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.[1,3,4,5] Figure 2.2. graphically relates these factors to plate height (H).[5]

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 differ. Solute molecules therefore reach the end of the column over a time interval, which leads to peak broadening. This effect, also called eddy diffusion, is directly proportional to the diameter of the packing particles. Multi-path peak broadening may be partially offset by ordinary diffusion, which results in the transfer of molecules from a stream following one pathway to a stream following anther pathway. At very low velocities, a large number of these transfers occur so that numerous pathways are sampled by each molecule and the rate at which each molecule moves down the column tends to approach the average. At moderate to high velocities, sufficient time for diffusion averaging is not available and band broadening is observed.[1,3]

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 the center). The longitudinal diffusion term is directly proportional to the mobile phase diffusion coefficient, D_M, and inversely proportional to the mobile phase velocity. Longitudinal diffusion is less pronounced in liquid chromatography as diffusion coefficients in liquids are several orders of magnitude smaller than those in gases.[1,5]

Band broadening resulting from mass-transfer effects arise 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 directly proportional to the velocity of the mobile phase as a fast flow rate leaves less time for equilibrium to be approached.[1,5]

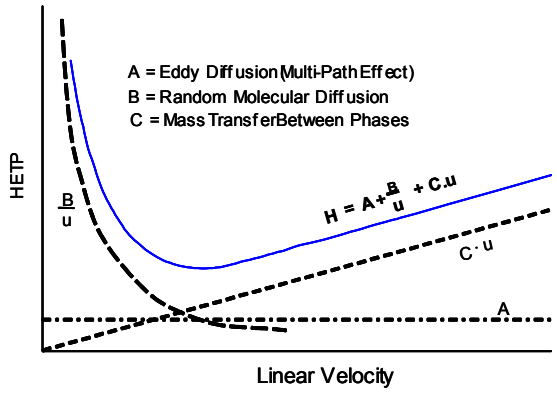

Figure 2.2.: The effects of A, B and C terms on plate height, H.*

* Introduction to Separation Science, Stellenbosch University 2007, P. Sandra, A.J. de Villiers.

2.2.3. Optimization of chromatographic resolution

A chromatographic separation is optimized by varying experimental conditions until the components of a mixture are separated efficiently with a minimum expenditure of time. In seeking optimum conditions for achieving a desired separation, the fundamental parameters pertaining to retention (k'), selectivity (α), and efficiency (N or H), may be adjusted. Optimization experiments are therefore aimed at altering the relative migration rates of solutes and at reducing peak broadening.[1]

Peak resolution, R_s, as expressed in terms of α, k' and N may therefore be optimized by manipulating each of these variables (equation 5).

Peak resolution is proportional to the square root of the plate number. A fourfold increase in the plate number therefore doubles the peak resolution. Resolution may therefore be optimized by increasing the plate number or by optimizing the plate height. The plate number may be increased by using longer columns while the plate height may be reduced by using smaller diameter columns in gas chromatography or by reducing the particle size in liquid chromatography. The plate height may also be reduced by optimizing the mobile phase velocity.

Optimization of the selectivity factor has the largest effect on resolution. Selectivity may be optimized by changing the stationary phase in gas chromatography while the stationary as well as the mobile phases may be altered in liquid chromatography to optimize selectivity.

For values above 5, the influence of the retention factor, k, on resolution is small. On the other hand, low k values result in poor peak resolution. In gas chromatography the retention factor may be increased by decreasing the column temperature, changing the stationary phase or increasing the phase ratio, while

in liquid chromatography the mobile phase, stationary phase or phase ratio may be altered to optimize the retention factor.[1,4]

2.2.4. Differences between liquid chromatography and gas chromatography

Focusing on liquid and gas chromatography, the most prevalent forms of chromatographic separations, some pertinent differences can be highlighted.

The high diffusion rates and low viscosity of gaseous separations inherently lend them to the use of capillary columns. Thin columns (commonly 250-320 μm i.d.) coated with a thin layer of liquid stationary phase are therefore used in modern gas chromatography. In liquid chromatography, by comparison, analyte diffusion and mobile phase viscosity are two orders of magnitude lower and higher, respectively, compared to gas chromatography. As a consequence, packed columns are used in liquid chromatography, where small particles are used to reduce the diffusion distances. As a direct result, high pressures are required to force the highly viscous liquid mobile phase through a packed bed.

Moreover, in gas chromatography, the mobile phase is an inert gas and no interactions take place between the solute and the mobile phase, so that separation is essentially a function of interactions between the solute and the stationary phase. As separation is performed in the gas phase, the distribution between the two phases is significantly affected by the volatility of the analyte, and therefore by the temperature. In fact, in the absence of specific interactions with the stationary phase, as commonly occurs when using apolar phases, separation is primarily based on differences in vapor pressures, with analytes eluting in sequence of increasing boiling point. For this reason, temperature programming is commonly used to regulate retention in GC separations.

In liquid chromatography an interactive mobile phase is utilized, which provides an additional parameter for selectivity tuning as separation is the result of interactions of the solute with both the mobile and stationary phases. Accordingly, mobile phase gradients are most often used to control analyte retention in HPLC.

In the following sections column characteristics, separation modes and instrumental aspects will be discussed briefly for liquid and gas chromatography.[1,6,7]

2.3. High performance liquid chromatography

Early liquid chromatographic separations were performed in large diameter glass columns packed with relatively large diameter stationary phase particles. Decreasing the particle size of columns affected vast increases in efficiency but required sophisticated instruments operating at high pressure. High performance liquid chromatography, HPLC, is the term used to distinguish these modern instrumental liquid chromatographic techniques from classical gravity flow- and-thin layer liquid chromatography. HPLC is currently the most widely used analytical separation technique, mainly as a result of its broad applicability and amenability to accurate and sensitive analysis.[1]

2.3.1. The HPLC column

The heart of the HPLC system is the column, where the actual separation of sample components occurs. Modern HPLC columns consist of heavy-walled stainless steel tubes (Figure 2.3.) pressure packed with fine-diameter packing material, and equipped with inlet and outlet fittings for incorporation into the system between the injector and detector.[8]

Macroporous silica gel is by far the most important adsorbent for liquid-solid chromatography and is also the material used to prepare most bonded phase packings. Silica particles can be produced reliably with defined particle size, are physically stable at high pressures, porous to increase sample capacity and can easily be derivatized with different materials to tune the selectivity.[3]

Figure 2.3.: HPLC columns, guard column (disposable cartridge and holder) and connector.*

* Agilent Technologies Inc., Waldbronn, Germany.

2.3.2. Column efficiency in HPLC

Plate numbers for liquid chromatographic columns are an order of magnitude smaller than those encountered with gas chromatographic columns. This is largely due to the pressure drop in liquid chromatography, which limits the potential length of packed columns. Gas chromatographic columns, which may be as long as 60 m, provide a larger number of theoretical plates and superior efficiency. Nonetheless, with two interactive phases and a diversity of stationary phases, HPLC offers a variety of variables that may be manipulated to optimize separation selectivity (the α factor) to separate complex mixtures.[1]

Adjustment of the solvent composition, in terms of the types of solvent as well as their relative proportions in the mobile phase, permits manipulation of k' and α to resolve the components in a mixture. The resolution may also be improved by increasing N, by lengthening the column or more practically, by reducing H. H may be reduced by reducing the particle size or by altering the flow rate (to operate at u_{opt}) or viscosity of the mobile phase. Reducing the viscosity affects an increase in the diffusion coefficient in the mobile phase and may be affected by elevating the column temperature. Finally, the nature of the interactions with the stationary phase may be changed by utilizing any of a variety of different stationary phases.[1]

Important variables that affect on-column peak broadening in liquid chromatography are the diameter and size distribution of the packing particles, diffusion coefficients in the mobile and stationary phases and the flow rate of the mobile phase.[1,8] The Van Deemter equation may be used to relate the parameters that causes peak broadening to H as a function of mobile phase velocity, and therefore to optimize these parameters.

2.3.3. Modes of separation in liquid chromatography

All forms of liquid chromatography are differential migration processes where sample components are selectively retained by a stationary phase. Retention in liquid chromatography is a complex process involving solute interactions in both the mobile and stationary phases. Modes of interaction include liquid-liquid (partition) chromatography, liquid-solid (adsorption) chromatography, ion exchange chromatography and two types of size exclusion chromatography namely gel-permeation and gel-filtration chromatography.[1,3]

Reversed phase liquid chromatography (RP-LC) is a form of partition chromatography which is based on the partitioning of analytes between a relatively apolar stationary phase and a relatively polar mobile phase and, is the most widely used separation mode in liquid chromatography. The popularity of reversed phase liquid chromatography is explained by its unmatched simplicity, versatility and scope. The near universal application of reversed phase liquid chromatography stems from the fact that virtually all organic molecules have hydrophobic regions in their structure and are capable of interacting with the stationary phase. The rapid equilibration of the stationary phase with changes in mobile phase composition ensures amenability with gradient elution techniques.[3] Reversed phase chromatography is most likely to provide optimum retention and selectivity where the solutes have limited hydrogen-bonding groups or a predominant aliphatic- or aromatic character. This mode of liquid chromatography is particularly well suited for separating solutes based on the size and structure of alkyl groups and was therefore used exclusively in the separations performed in this study.[3,6] The following discussion will therefore be limited to reversed phase liquid chromatography.

Retention in reversed phase liquid chromatography occurs by non-specific hydrophobic interactions of the solute with the stationary phase. Hydrophobic retention, as encountered in RP-LC, involves mainly apolar compounds or apolar portions of molecules. 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. The predominant factors that determines the hydrophobicity of the stationary phase are the length of the alkyl chain or the total number of carbon atoms as well as the bonding density.[3,6,9]

In reversed phase liquid chromatography (RP-LC), the apolar, hydrophobic stationary phase is commonly obtained by chemical derivitization of silica particles with apolar moieties such as C18 functional groups. The mobile phase commonly consists of a partially or fully aqueous solution. Elution of the solute is

achieved by decreasing the polarity of the mobile phase through increasing the fraction of organic modifier. Under such conditions, the least polar, hydrophobic solutes are retained more strongly and display the longest retention times while polar compounds do not partition to a significant extent into the stationary phase and elute first. Methanol and acetonitrile are popular solvents because they have low viscosities, are readily available with excellent purity and are miscible with water in all proportions. Eluents of intermediate strength between these solvents and water are usually obtained by mixing these in appropriate ratios.[6]

Isocratic elution, where the composition of the mobile phase remains unchanged throughout the separation process, may fail to provide separation of compounds with widely varying k' values within an acceptable time. To separate a sample that contains compounds that are both weakly and strongly retained, the retention of the individual compounds must be changed during the chromatographic run, using a process called gradient elution. Gradient elution involves changing the mobile phase composition, either stepwise or continuously, during the chromatographic analysis. This process is used to gradually reduce k' values of the components so that all compounds elute without excessive analysis time (and band broadening). The small k' value at the time of elution affects narrow peakwidths, resulting in increased sensitivity. In reversed phase chromatography, solvent gradients are generated by increasing the fraction of organic modifier in the mobile phase.[6] Modern HPLC instruments may use up to four solvents to produce intricate gradients to allow separation of complex mixtures.

The stationary phase for C18 columns are typically prepared by covalent attachment of the octadecyl hydrocarbon to silanol groups on the silica support particles. Bonded-phase packings are relatively stable as the stationary phase is chemically bound to the solid support. The siloxane (Si-O-Si-R) bond, widely used for this purpose, is prepared by the reaction of alkoxysilane with surface silanol groups of hydrolyzed silica gel. For steric reasons, it is not possible for all

silanol groups to react and the silica surface consequently consists of a small percentage of un-derivatized silanol groups in addition to the hydrocarbon functional groups. Remaining silanol groups may be removed 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, and reduces 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 may also cause unwanted band broadening for basic compounds. The bonded alkyl phases offer fast analyses and rapid re-equilibration when the mobile phase is altered, as in gradient elution. The stability of the Si-O-Si bond is dependent the mobile phase pH and above pH 7, the bonds may be hydrolized with consequent degradation of the stationary phase.[1,6]

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, these materials may be used for reversed phase separations. Polystyrene-divinylbenzene phase may be used in the manner. The possibility of utilizing π–π interactions or charge transfer effects with the phenyl phase may lead to different selectivity than solely hydrophobic interactions. The phase is suited for non-selective retention of solutes of low to moderate polarity via a reversed phase mechanism. 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 may lead to non-reproducible chromatographic performance.[6,8,9]

Ion-pair chromatography, which may be considered to be a version of RP-LC, is used to separate ionized or ionizable species on reversed phase columns. In ion-pair chromatography an ion-pairing reagent is added to the mobile phase. The

ion-pair reagent possesses both hydrophobic and polar (typically ionic) functional groups and as a result is retained by the stationary phase, thereby providing the otherwise apolar stationary phase with an ionic functionality. Most ion-pair reagents contain alkyl groups to enhance retention on the apolar stationary phase. The modified stationary phase may then retain and separate organic solute ions of opposite charge by formation of a reversible ion-pair complexes. At the same time, the stationary phase is able to retain and separate non-ionized organic solutes. Ion-pair chromatography thus extends the utility of reversed phase chromatography to ionic compounds.[6]

Sample focusing is readily adaptable to reversed-phase chromatography. An injection solvent such as water that is significantly weaker than the mobile phase is used to dissolve the sample. Focusing of the solute plug occurs at the head of the column as the retention of analytes is enhanced under these conditions. Gradient elution may subsequently be used to separate the sample starting from a narrow band.[6]

2.3.4. HPLC instrumentation

An HPLC separation is performed by pumping the mobile phase from a reservoir, through an optional degasser and injector, into the column, and out to the detector. The sample, dissolved in a suitable solvent, is introduced into the flowing mobile phase by making use of the injector. Inside the column, separation of analyte molecules occurs according to the specific column and mobile phase combination, following which analytes are eluted from the column and passed into the detector. The detector generates a signal for the separated compounds (if detected), which is sent to a suitable data system. The data system then generates a plot of signal intensity as a function of time – this 'picture' of the separation is called a chromatogram. Figure 2.4. presents a schematic representation of an HPLC system. Chromatographic data may be quantitative

and where the detector is capable of acquiring spectral information of eluted compounds, qualitative data may be generated at the same time.[8] An overview of the hardware that supports the column in HPLC will be presented with special reference to the equipment used in this study.

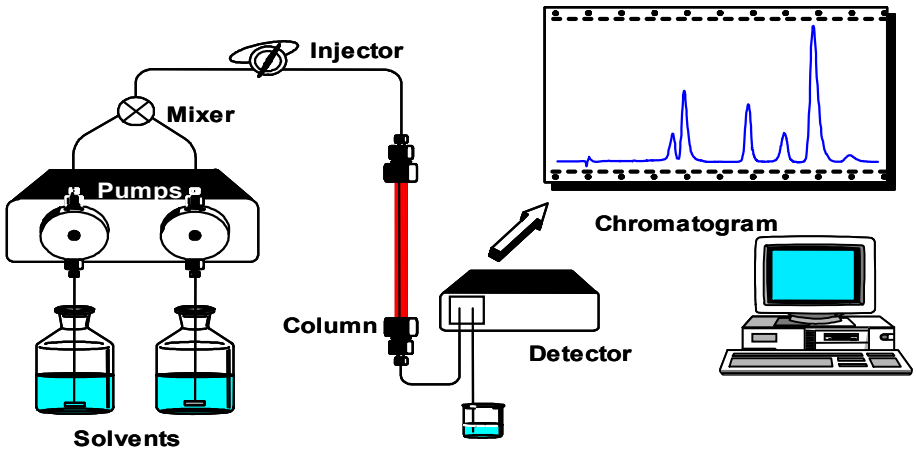

Figure 2.4.: Schematic of HPLC instrument.*

* Introduction to Separation Science, Stellenbosch University 2007, P. Sandra, A.J. de Villiers.

2.3.5. Mobile phase treatment system

HPLC instruments are usually equipped with glass reservoirs that contain the solvents. Dissolved gasses, mostly oxygen and nitrogen, may interfere with the analysis by forming bubbles in the system. Bubbles may cause the pump to malfunction, lead to band spreading and interfere with the performance of the detector.[1]

Dissolved gasses may be removed by any of a number of strategies. Degassing may be achieved by sparging, where gasses are swept out of solution by fine

bubbles of an inert gas that is not soluble in the mobile phase. Helium is often used for this purpose. Continuous vacuum degassers use permeable membranes that separate the mobile phase stream from a vacuum source, thereby effectively outgassing the mobile phase before it is fed into the pumping system. Suction filtration also effectively removes dissolved gasses from the mobile phase in addition to elimination of suspended particles. Degassing of the mobile phase may also be achieved by sonication, distillation or heating and stirring.[1]

2.3.6. Solvent delivery system

The solvent delivery system acquires solvent from the reservoirs through a filter, pressurizes the solvent sufficiently to overcome resistance from the column packing material and drives it through the system. The demands placed on an HPLC solvent delivery system are severe. These include the requirements to produce a reproducible pulse-free output at pressures up to 400 bar with accurate flow control at flow rates from 0.1 to 10 mL/min. For gradient elution precise and accurate control over at least two solvents is desired. The system should also have a small internal volume for solvent variation in gradient elution and all components should be corrosion resistant.[1]

Basic pump designs include the reciprocating piston displacement pump, syringe pumps and pneumatic or constant pressure pumps.[1] As the reciprocating piston displacement pump is used almost exclusively in modern solvent delivery systems, the discussion will be limited to this design.

This pump consists of a metal body drilled out to provide a pumping chamber that is sealed at the back with a Teflon seal through which an inert piston rides. Pistons are commonly made of beryl glass and are operated by a cam-driven motor. The inlet and outlet ports are equipped with check-valves that open and

close alternately to control the flow of solvent into and out of the cylinder. During the filling stroke, the inlet check-valve opens to admit solvent to the chamber while the outlet valve closes to prevent pressurized solvent from re-entering the chamber. During the pressurization stroke the inlet check-valve closes to prevent solvent from running back into the reservoir while the outlet valve opens to allow solvent delivery to the system.[8]

A problem inherent to this design is that pressure pulses are produced which are transmitted through the system and negatively affect the analysis in the form of signal noise. This problem may be minimized by three methods.[8] Opposing multiple pump heads may be used to pressurize the system at different times so that at any particular time one or the other pump is pressurizing the system. By arranging the pressurizing cycles of the two pumps so that they are slightly out of phase, a gradual take-over between the alternate delivery strokes may be established.[6] This method increases the cost of the solvent delivery system, but produces relatively pulse-free solvent delivery. The system can also be designed to speed up the refilling stroke with the result that the single pump spends the majority of its time in the delivery mode. This design is not as efficient as dual-pump systems, but is less expensive. Pulse dampeners may also be used in conjunction with dual-pump or single pump systems to reduce pressure pulsation in the solvent delivery system.[8]

A separation that employs a single solvent of constant composition is called an isocratic elution. Separation efficiency may be enhanced by gradient elution where the solvent delivery system is programmed to mix two or more solvents, continuously or in a series of steps. Solvents used in the formation of the gradient may be mixed in two ways, after the pump-heads (high pressure mixing) or before the pumps (low pressure mixing). High-pressure mixing essentially combines the output of two isocratic pumps, each dedicated to one solvent. This system is expensive and essentially restricted to binary gradients, but can produce sharp, reproducible gradient profiles. Low-pressure mixing employs a

proportioning valve between the pump head and the solvent reservoirs and is capable of mixing more than two channels, but efficient degassing is required for accurate formation of the gradient.[6,8]

A small internal volume is critical in pump design as changes in solvent composition during gradient elution should be rapid and accurate. The capacity of the pump chamber in most reciprocating piston displacement pumps vary from approximately 35 to 400 µL which makes this design amenable to gradient elution techniques.[6]

2.3.7. Sample injection systems

The main criteria for sample introduction in HPLC are good precision of injection volumes, low memory effects, and the ability to inject variable sample volumes. The injection system should also possess minimal dead volume so that negligible peak broadening is contributed by injection. Sample introduction may be performed manually or by using an automated injector. Automated sampling systems offer advantages such as better reproducibility and increased throughput. Modern automated sampling systems may also offer features such as on-line pre-column derivatization, dilution of samples, internal standard addition, barcode readout for sample tracking and heating or cooling for improved stability.[1,6,8,10]

Injection systems are commonly based on a six-port valve, which is switched between two positions for each injection to permit sample introduction without depressurizing the system (Figure 2.5.). During the loading phase, the sample is drawn or placed into a sample loop. When the valve is switched to the inject position, the loop becomes part of the mobile phase stream, thus transporting the sample to the column. Most automated injectors utilize a metering device based on a precision syringe to inject volumes ranging from 0.1 to 1800 µL, depending

on the configuration of the system. With this design, all parts of the system are constantly flushed during the analysis so that possible remaining sample components are effectively removed, thereby reducing carry-over.[1,6,10]

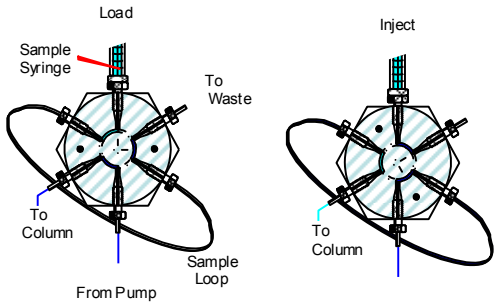

Figure 2.5.: Six-port sampling valve with fixed volume loop.*

* Introduction to Separation Science, Stellenbosch University 2007, P. Sandra, A.J. de Villiers.

2.3.8. Column thermostat

Variations in temperature may cause changes in retention time and affect the precision of quantitative measurement. Elevated temperature may be advantageous because decreased mobile phase viscosity, increased mass transfer and increased sample solubility may result in better resolution and faster analysis. Temperature control may therefore enhance the efficiency and reproducibility of chromatographic separations.[8]

Most modern HPLC instruments are equipped with column compartments that allow column temperature control. The mobile phase may be pre-heated before

entering the column. Temperatures may be regulated to a few tenths of a degree from near-ambient to 200°C.[1]

2.3.9. Detectors

The ideal detector for liquid chromatography should be sensitive and selective and characterized by a linear response to the solute over a wide dynamic range. In addition 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 high-efficiency, small-particle columns, the detector should also have a fast response time.[1]

Liquid chromatographic detectors can be divided into two basic types. Bulk property detectors respond to a mobile phase bulk property, such as refractive index. In contrast, solute property detectors respond to a property of the solute, such as UV absorbance or fluorescence. The bulk property detectors are universal but are relatively insensitive compared to solute property detectors. Pre- and post-column derivatization techniques may be used to expand the applicability of the solute property detectors.[6] The most common detectors used in combination with liquid chromatography include.[1]

- Absorbance
- Fluorescence
- Electrochemical
- Refractive index
- Conductivity
- Mass spectrometry
- FT-IR
- Light scattering

The most common liquid chromatographic detectors in use are based upon UV-visible absorption. These optical detectors are classified as fixed-wavelength, variable wavelength or diode array detectors.[6] For the purposes of this study, diode array and mass spectrometric detectors will be discussed.

2.3.9.1. Diode array detector

Ultraviolet frequencies correspond to wavelengths of approximately 10 to 400 nm while the visible spectrum extends from approximately 400 to 750 nm.[11] UV-visible energies correspond to electronic transitions, the energy needed to excite an electron from one molecular orbital to the next.[12] During transition to a higher electronic level, a molecule can undergo simultaneous transitions between any of a number of sub-levels, corresponding to various vibrational and rotational states. As a result UV-visible absorption bands are broad and the corresponding spectra are lacking in detail.[13,14] Absorption by molecular oxygen limits the range of conventional UV-visible detectors to wavelengths longer than approximately 190 nm.[12]

For absorbance measurements in the eluent from a chromatographic column, a flow-through cell that is typically based on a Z-shaped design is used. In order to minimize extra column band broadening, the volume of the cell is kept as small as possible while at the same time maintaining a sufficient path length. Typical cell volumes are of the order of 10 µL per centimeter of optical path length for conventional diameter separation columns. The energy that passes through the flow cell is compared with that of a reference and a signal that is the log of the ratio of the two measurements is produced. In accordance with the Beer-Lambert law, the absorption of monochromatic radiation is linear with solute concentration.[1,3]

The Beer-Lambert law is typically given by the following equation:

$$A = \log (I_0 / I) = \varepsilon b c \qquad (10)$$

where I_0 and I is the incident and transmitted intensities, ε is the molar absorptivity of the solute with units of $L.mol^{-1}.cm^{-1}$, b the path length in centimetres and c the molar concentration of the solute.[1] This relationship predicts that the magnitude of the absorption signal is proportional to the molar absorptivity of the solute at the particular wavelength, the pathlength of the detector and the solute concentration.

The most powerful ultraviolet-visible spectrophotometric detectors are diode array instruments, which permit simultaneous collection of data over the wavelength range of approximately 190 to 900 nm. These detectors are equipped with an illumination source that is typically a combination of deuterium-arc-discharge lamp for the UV radiation and a tungsten lamp for the visible region. Diode array detectors work in a parallel configuration by simultaneously monitoring all wavelengths. Energy from the sample cell is focused onto a dispersion device, typically a grating, and the resulting monochromatic wavelengths are directed onto the array of photodetectors (Figure 2.6.). Complete spectra may thus be obtained in fractions of a second.[3,6,15]

Spectral data may be used to elucidate qualitative information from chromatographic data in addition to the customary quantitative information. The ratio of the absorbance at two wavelengths is structure specific and the purity of a chromatographic peak may be elucidated by absorbance ratio-ing, a technique whereby the ratio of absorbance at two wavelengths in the spectra is compared. Analyte absorption spectra may also be compared with a library of user-generated spectra for tentative compound identification.[6]

For UV detection, mobile phase solvents should be chosen to ensure low absorbtion at the wavelength region of interest. Water, methanol and acetonitrile

permit operation in the UV range down to 210 nm. Where the acquisition wavelengths are chosen so that the mobile phase does not absorb strongly, UV-visible detectors can be used with gradient methods.[6]

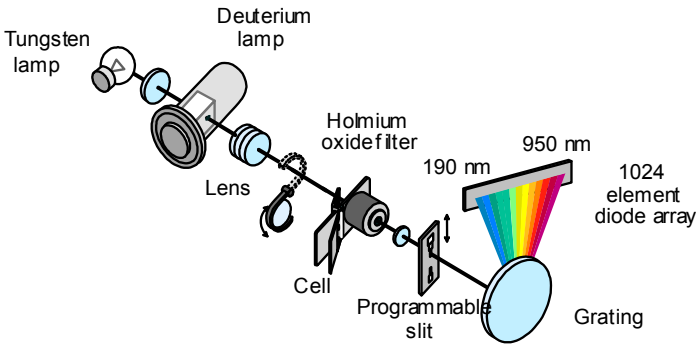

Figure 2.6.: Components of a UV visible diode array detector.*

* Agilent Technologies Inc., Waldbronn, Germany.

2.3.9.2. Mass spectrometric detector

Mass spectrometry, one of the most generally applicable of all analytical tools, provides qualitative and quantitative information about the atomic and molecular composition of inorganic and organic materials. The main advantages of mass spectrometry as an analytical technique are increased sensitivity and specificity compared to most other analytical techniques. The sensitivity and specificity results primarily from a combination of the action of the analyzer as an effective mass-to-charge ratio filter (thereby reducing background interference), sensitive

electron multiplier detectors and characteristic fragmentation patterns of solute molecules.[6] For these reasons, the mass spectrometer is probably the closest to the ideal detector for liquid chromatography. Interfacing liquid chromatography with mass spectrometry provides a powerful analytical technique particularly amenable to trace level analysis. A schematic diagram of the components of a typical liquid chromatography-mass spectrometry instrument is depicted in Figure 2.7.

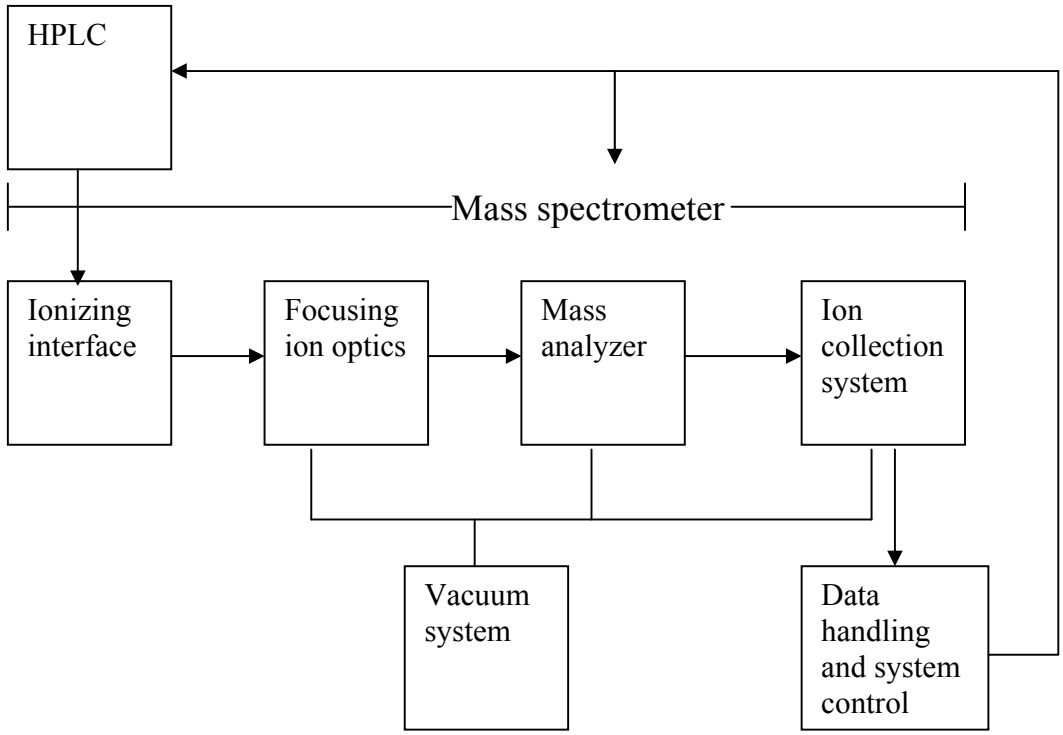

Figure 2.7.: Components of an LC-MS system.[2]

A fundamental problem in coupling liquid chromatography with mass spectrometry is the enormous mismatch between the relatively large mass flows involved in HPLC and the vacuum requirements of the mass spectrometer.[1] To overcome this problem, several interfaces have been developed for efficient sample introduction into the mass spectrometer.

Modern LC/MS systems generally utilize non-evacuated interfaces in which the solvent is removed as the sample is ionized prior to introduction into the high-vacuum environment of the mass spectrometer. Such interfaces mainly comprise a combination of heaters, reduced pressure and gas nebulizers to volatilize and remove unwanted eluent. Concurrent ionization of target compounds is achieved by spraying the eluent either from an electrically charged capillary or across a corona discharge needle during the final stages of evaporation. Generally the configuration may be changed to produce positively or negatively charged ions. The two prevalent types of atmospheric pressure interfaces that are also relevant to the current study are the electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces. Each of these may fail to ionize some types of compounds and switching between sources may be required in order to ionize as many as possible of the sample components. Multimode sources capable of ionizing diverse compounds are available but this design suffers from a loss in sensitivity compared to dedicated sources.[8]

2.3.9.2.1. Electrospray interface

The electrospray ionization (ESI) interface (Figure 2.8.) is suited for use with polar and ionized materials. It is a soft ionization technique that is characterized by little fragmentation. It is also concentration independent and therefore suited for use with microflow liquid chromatography systems.[8]

In the electrospray interface ionization is accomplished by passing the effluent from the liquid chromatograph down a heated metal capillary tube along which a 3 to 4 kV electric charge differential is applied. This field charges the surface of the liquid and forms a spray of charged droplets. The evaporating liquid is sprayed out of the end of the tube where a concentric flow of an inert nebulizing gas aids final evaporation. The charged droplets are attracted towards a capillary sampling orifice while a counterflow of heated drying gas shrinks the droplets and

carries away uncharged material. The droplets shrink to the point where electrostatic forces exceed the cohesive forces and the ions are desorbed into the gas phase. The gas phase ions pass into the low-pressure region of the ion source.[3,8,16]

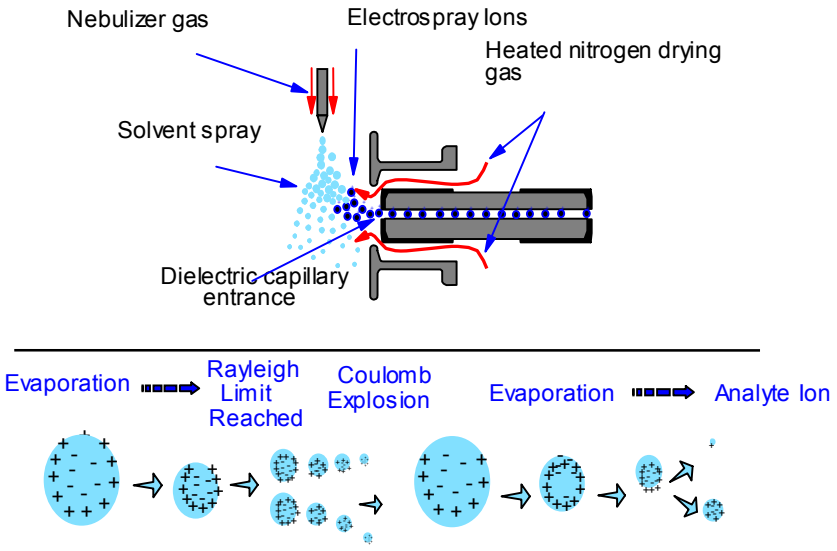

Figure 2.8.: Electrospray interface and schematic ESI process.*

* Agilent Technologies Inc., Waldbronn, Germany.

2.3.9.2.2. Atmospheric pressure chemical ionization interface

The atmospheric pressure chemical ionization (APCI) interface is typically used for less polar compounds and can accommodate relatively large flow rates. As with ESI, the design utilizes an off-axis flow path to ensure that only the charged sample components enter the mass spectrometer while mobile phase solvent is diverted out of the system. The APCI interface utilizes an inert nebulizing gas to entrain and break up the eluent stream into small droplets. The nebulizer capillary may be heated to aid evaporation of the solvent. After the desolvation

process, a dry vapor of solvent and analyte ions are produced which are sprayed across a corona discharge needle operated at approximately 25 kV to ionize the shrinking droplets. Solvent molecules are then ionized by the corona discharge and act as an ionized reagent gas which subsequently ionizes analyte molecules by atmospheric pressure chemical ionization. A cone-shaped impactor plate, pointing towards the source region, is charged opposite to that applied to the corona needle to draw the ions to the mass spectrometer entrance. A countercurrent flow of dry nitrogen gas acts as a curtain to sweep uncharged solvent vapors away from the pinhole orifice thus minimizing clustering of charged analyte ions with water and other polar molecules.[3,8] Figure 2.9. presents a schematic representation of the APCI process.

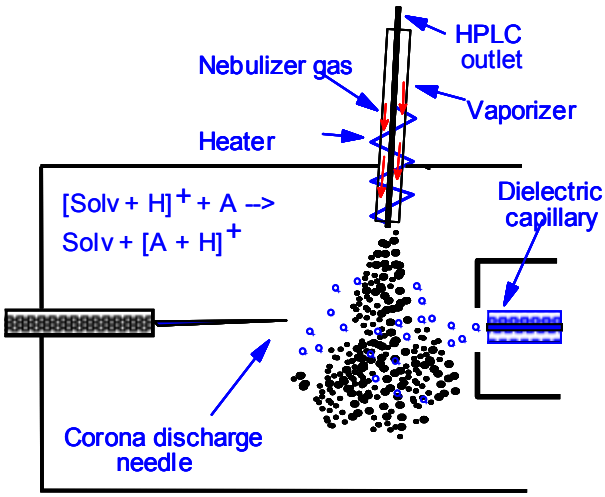

Figure 2.9.: Atmospheric pressure chemical ionization interface and schematic APCI process.*

* Agilent Technologies Inc., Waldbronn, Germany.

2.3.9.3. Ion optics

Once a sample is ionized, the charged ions are moved from the interface through a pinhole entrance into the higher-vacuum area of the spectrometer. An oppositely charged draw-out lens works with movement from atmospheric pressure to high-vacuum to pull ions into the entrance chamber. A repeller at the back of the entrance has the same charge as the ions and forces them into the focussing lens. Variable voltage charges on the focusing lens with the same polarity as the molecular ions function by squeezing the ions into a narrow stream that is focussed on the mass analyzer.

2.3.9.4. Mass analyzer

Functionally, all mass spectrometers perform three basic tasks; creating gaseous ion fragments from the sample, sorting these ions according to their mass-to-charge (*m/z*) ratios and measuring the relative abundance of the ion fragments of each mass.[6] LC-MS systems may be categorized into four basic designs according to the mass analyzer system used. The quadrupole (or octapole) type analyzer use a bundle of oppositely charged rods, swept with radio frequency, to separate and focus the ions on the detector. In the ion trap design, a three dimensional spherical or quadrupole arrangement is used to contain and then release the ions. Separation is achieved by varying the dc/RF voltage to expel ions of increasing *m/z* into the detector. The third type ejects ions in a burst mode into a time-of-flight tube where the traveling time to the detector is dependent on the *m/z* ratio of the ions, with lighter fragments arriving at the detector first. The fourth type has a trapping volume that is bombarded with a full frequency radio signal that momentarily promotes ions to a higher-energy state. Upon collapse a modified all-frequency signal is produced which is analyzed with Fourier transform software to produce an intensity versus *m/z* ratio signal.[8]

Combinations of one or all of these designs are used to create hybrid systems that combine multiple mass analyzer modules with collision and ion-trapping cells to separate, fragment and detect not only the molecular ion, but also fragments, commonly referred to as daughter ions. The ability of these tandem MS or MS/MS systems to determine fragmentation products of particular molecular ions in this manner allows the analyst to perform structural elucidation studies or to confirm the identity of the solute. Hybrid systems are also suited for accurate quantitation at trace levels as the analyzers can be tuned to single ion frequencies corresponding to the molecular ion and daughter ion of the target compound, thus generating very specific signals with low background noise.[8] The focus of the following discussion will be on the triple quadrupole mass spectrometer design, utilized in this study.

2.3.9.4.1. Triple quadrupole mass analyzers

The triple quadrupole mass spectrometer combines two conventional scanning quadrupole analyzers with a collision cell. The target ion selected from the first analyzer is allowed to collide with inert gas molecules in the collision cell to induce fragmentation. The fragmented ions are then passed into the second analyzer for separation and subsequent detection. In earlier models, a quadrupole was used as a collision cell hence the term triple quadrupole mass spectrometer.[8] To identify these components in the following discussion, the first quadrupole analyzer, collision cell and second quadrupole analyzer will be numbered Q1, Q2 and Q3, respectively.

The quadrupole analyzer has four cylindrical rods clamped in a tubular arrangement by ceramic collars. The exact hyperbolic spacing between diagonally opposed rods is critical for mass spectrometer operation. Opposing rods have the same direct current charge applied to them while adjacent rods have opposite charge applied. Ions are focussed into the tunnel formed by the

four rods and follow a corkscrew flight path down the rods as they are swept forward by a changing radio frequency signal. Only ions following stable trajectories at a given frequency are expelled from the quadrupole to collide with the ion detector. Ion masses that follow unstable paths at the particular dc/RF voltage collide with the inner walls of the analyzer rods, and are not detected.[8]

Two scanning techniques, continuous scanning and selected ion monitoring (SIM) are used to acquire mass spectrometric data. In the continuous scan mode, a scan of a pre-defined mass range is performed at fixed time intervals for the duration of the experiment. A total ion current profile may thus be generated that represents a normalized plot of the sum of ion abundance as a function of time. Mass spectra may then be obtained for each of the sequence of scans thus performed. When operated in SIM mode, one or a few ions may be monitored exclusively throughout the experiment.[3]

Following ionization and ion focusing, ions enter Q1, where the direct-current charged surfaces of the analyzer are then swept with a changing radio frequency signal that selects different mass ions for each frequency and allows them to follow a stable path through the analyzer. The stable ions at each frequency are then expelled into the collision cell Q2. Here, selected parent ions are fragmented by collision with molecules of an inert gas. The energy of these collisions, and therefore the degree of fragmentation that the molecules undergo, is regulated by the collision voltage. The resulting daughter ions are then focussed onto the second quadrupole analyzer, Q3. The mass spectrum of the resulting daughter ions is obtained by scanning with quadrupole analyzer Q3. In this manner a complete three-dimensional fragmentation map may be obtained by recording the mass spectrum of each fragmentation ion of a parent compound.[8]

The triple quadrupole LC-MS may be operated in any one of four modes depending on the aim of the experiment. In the parent ion mode, Q1 is scanned

and all ions sent to the fragmentation chamber. Quadrupole Q3 is then 'parked' at a frequency to select a specific fragment ion common to related compounds. In the daughter 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. The fourth operational mode is particularly suited for the trace analysis of compounds in complex mixtures, even in cases where the components are not completely separated. In this mode, both Q1 and Q3 are set at single ion frequencies specific for the compound (ion) under investigation and one of its daughter fragments, respectively. The signals that are generated are therefore very specific to the compound under investigation 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 and time, thus producing a signal with higher signal-to-noise ratios. In this way sensitivity and detection limits may be optimised.[8,18]

2.3.9.5. Ion detectors

The resolved ion beam, after passage through the mass analyzer, sequentially strikes the ion detector. Several types of detector are used with mass spectrometers. For ion currents less than 10^{-15} A, an electron multiplier detector is required.[6] This discussion will focus on the electron multiplier detector.

The electron multiplier detector uses an impact and cascade mechanism. When a charged particle strikes the membrane surface of the conversion dynode, the impinging ions are converted to electrons. The resulting electrons then strike the coated surface of the detector wall, releasing multiple electrons. These electrons

in turn cascade down the curved tube of the detector, releasing multiple electrons on each impact. This cascade of electrons amplifies the signal of a single contact for transfer to the data system. Gain ranges of the order of 10^5 to 10^7 may be attained with this detector design.[6,8,17]

2.3.9.6. Vacuum system

The operation of the spectrometer requires a collision-free path for the ions. To achieve this, the pressure in the spectrometer should be less than 10^{-6} torr to prevent collisions of the ions with air molecules.[6,8]

The required high-vacuum environment is established in a two-staged process. A rough vacuum is established first with a laboratory rotary-vane oil pump. A turbomolecular pump connected in series provides the final operating vacuum. The turbo pump has a series of vanes mounted on a shaft that rotates at very high speeds. The vanes force the air from the vacuum chamber prior to venting it out through the roughing pump. The turbo pump contains no oil that can contaminate the analyzer, but may be prone to mechanical failure.[8]

2.3.9.7. LC-MS data handling

The data system may be used to record the signal strength of all ions detected as well as spectral information at any given time in the chromatographic run in a three dimensional block of data. A total ion chromatogram (TIC) is a plot of the signal strength of all ions versus time whereas a spectrum is a plot of signal strength versus mass to charge ratio at any given time. The *m/z* ratios of the ions are determined by calibrating the dc/RF frequency with standards of known mass to charge ratio.[8]

2.4. Gas chromatography

Gas chromatography is in many instances the technique of choice for the separation of thermally stable and volatile organic and inorganic compounds. In gas chromatography, the sample, often vaporized, is injected onto the head of a chromatographic column. As with HPLC, the column is where the actual separation of sample components occurs. Elution is brought about by the flow of an inert gaseous mobile phase. The mobile phase transports the gaseous analyte through the column and separation, specific to the column and analyte combination, occurs prior to their detection. Gas-liquid chromatography, based upon the partitioning of the analyte between a gaseous mobile phase and a liquid stationary phase which is immobilized on the surface of an inert solid, is by far the most widely used form of gas chromatography.[1,6] This discussion will focus exclusively on gas-liquid chromatography. An overview of relevant gas chromatographic techniques and hardware is presented with special reference to the equipment utilized in publications referenced in this study.

2.4.1. Gas chromatographic columns

Two general types of columns are encountered in gas chromatography, namely packed and capillary columns. Capillary columns provide faster and more efficient separation and are almost exclusively used today. Capillary columns are usually constructed of fused silica and may be as long as 50 m or more. The high permeability or low resistance to carrier-gas flow of capillary columns enables long columns to generate large numbers of theoretical plates.[2,6] In order to fit into an oven for thermostatting, capillary columns are formed into coils of suitable diameter.

In capillary columns the stationary phase is a uniform film of liquid with thickness of the order of a few tenths of a micrometer coated on the interior of the capillary. Film thickness primarily affects the retentive character and capacity of a column. Desirable properties for the immobilized liquid stationary phase includes low volatility, thermal stability, chemical inertness and solvent characteristics such that k' and α values for the solutes to be resolved fall within a suitable range. The criteria for column efficiency, described earlier for HPLC, are fully applicable to gas chromatography. Two differences should be noted however. First, there is no contribution to band broadening due to eddy diffusion in capillary columns. Second, longitudinal diffusion is more important in gas chromatography as diffusion rates in gasses are much larger than that in liquids.[1]

Retention time for a solute in a given column depends upon its distribution constant, as discussed earlier. In GC this distribution is dependant on the volatility of the analyte, and therefore the temperature, as well as on chemical interaction with the stationary phase. Two types of separation can be distinguished. In the first instance, when an apolar stationary phase such as polydimethylsiloxane (PDMS) is used, non-specific interaction with the stationary phase dominates. Analytes are therefore separated largely due to differences in volatility. This is determined by the respective vapor pressures of the analytes, and this mode of separation is referred to as a boiling point separation (analytes elute in order of increasing boiling points). In contrast, when more polar stationary phases such as polyethylene glycol (PEG) are used, specific chemical interactions such as dipole interactions and hydrogen bonding predominantly determine analyte retention. A range of stationary phases, varying from relatively apolar to relatively polar, are available to achieve satisfactory separations.[1]

2.4.2. Carrier gases

Chemically inert carrier gases that are most often used in gas chromatography include helium, nitrogen and hydrogen. Associated with the gas supply are pressure regulators and gauges and optional purifiers to remove impurities and moisture. Flow rates are controlled by pressure regulators in the instrument. Modern gas chromatographs are often equipped with electronic flow meters that enable constant flow or constant pressure modes of operation.[1]

The purpose of the carrier gas is to transport the sample through the column to the detector. The three most common gases provide practically the same minimum plate height, but the optimal linear velocities increase in the order nitrogen < helium < hydrogen. Plate height curves for helium and hydrogen are flatter meaning that a smaller loss in efficiency will be suffered when linear velocities higher than the optimum are used, leading to significant reductions in analysis times. The ratio of carrier gas viscosity to the diffusion coefficients of the sample components should be as small as possible and in this respect hydrogen, followed by helium are the most efficient carrier gasses.[6]

2.4.3. Sample injection systems

In order to effectively exploit the high efficiencies of GC columns, it is required that a suitable sample size be introduced as a discrete band of vapor. The most common method of sample injection involves the use of a microsyringe to inject a liquid or gaseous sample through a self-sealing septum into a vaporizer port, located at the head of the column. The injection port is usually maintained at about 50°C above the boiling point of the least volatile component of the sample. Capillary columns, commonly used today, require smaller sample sizes than that produced by a microsyringe. A split injector is employed to deliver a fraction of the injected sample to the head of the column, with the remainder going to waste.

Splitless injection may be utilized for trace-level analysis where the entire sample, including any solvent, is injected onto the column (this method often requires focusing mechanisms to avoid excessive band broadening due to injection). The injection port is vented after a predetermined amount of time to eliminate excess solvent from the system. Alternatively, although less often used, more reproducible injection of liquids and gases may be achieved by using a six-port rotary sample valve, similar to that used with liquid chromatography.[1,6]

Headspace sampling is a technique that may be utilized when only the vapor above the sample is of interest, particularly if the sample is a liquid that would normally require some processing before injection, such as blood. Headspace sampling can be done on any sample provided that the partition coefficient allows a sufficient amount of analyte into the gaseous phase.[6]

Purge and trap may similarly be used to purge volatile organic constituents from a sample which may then be trapped on a suitable adsorbent. Efficient desorption may be achieved by purging with an inert gas at elevated temperature (~300°C). Desorbed volatiles are typically re-focused by cryo-trapping. The gas chromatographic separation may then proceed upon heating and elution of the volatiles onto the chromatographic column.[6]

Gas-liquid chromatography reaches a practical limit when the amount of energy needed to vaporize the sample is the same as the amount of energy required to break a carbon-carbon bond. The technique of pyrolysis (or controlled fragmentation) extends gas chromatographic analysis to such low-volatility compounds as rubber and polymers and involves the formation of volatile fragments for subsequent introduction into the chromatographic column for analysis.[6]

Cool on-column injection is a capillary column inlet that allows direct deposition of liquid sample into the column. Both the inlet and the column are held

substantially below the boiling point of the solvent during the injection, after which both are increased to vaporize the sample and initiate chromatography. The temperature of the inlet should be controlled independently from the column temperature for highest reproducibility and flexibility. Since the sample is deposited directly into the column without being evaporated first, cool on-column inlets have the highest reproducibility and lowest discrimination and decomposition of any inlet. Sample introduction into small diameter capillary columns may however be problematic, and focusing methods generally are imperative in this mode of injection.[2]

Solid Phase Microextraction (SPME) is a form of sample preparation which involves exposing a polymer-coated fused silica fiber to a sample. The analytes partition into the stationary phase until an equilibrium has been reached, after which the fiber is removed from the sample. Analytes may then be thermally desorbed in the injector of a gas chromatograph. The fiber is typically contained in a syringe-like device to facilitate handling. SPME sampling may be performed directly in liquid samples or, if the analytes are sufficiently volatile, from the headspace above the liquid sample. In SPME analytes are not extracted quantitatively from the matrix, instead the extracted quantity depends on the equilibrium distribution of analyte between the sorbent and sample matrix. This approach requires careful calibration utilizing surrogates or standard addition to quantify the analytes and to compensate for matrix variations and the associated effects on distribution ratios.[19]

Automatic samplers may be utilized to provide unattended operation of most sample introduction techniques. Automatic samplers are consistently more precise than manual sample measurement and injection.[6]

2.4.4. Derivativization

Derivatization prior to gas chromatographic analysis is often desirable to improve thermal stability, adjust volatility or to introduce a detector-orientated tag into a molecule. Ideally all relevant functional groups should be derivatized quantitatively and rapidly and upon injection of the reaction mixture, the excess reagent should elute with the solvent peak.[6]

2.4.5. Column oven

Column temperature is an important variable, as outlined above, and must be controlled to a few tenths of a degree for precise GC work. The optimal column temperature depends on the boiling point range of the sample components and degree of separation required. In general, optimal resolution is associated with minimal temperature, but the cost of lower temperature is an increase in elution time.[1] Most often temperature gradients are used in capillary GC to effectively separate analytes across a broad range of boiling points with sufficient resolution in an acceptable time.

2.4.6. Detectors

As is the case with HPLC, a multitude of detectors have been developed for gas chromatography. The characteristics of the ideal detector are basically the same as that described for HPLC. Detectors available for use with gas chromatographic systems include the following:[1]

- Flame ionization detector
- Thermal conductivity detector
- Sulfur chemiluminescence detector

- Electron-capture detector
- Atomic emission detector
- Nitrogen phosphorous detector
- Flame photometric detector
- Mass spectrometric detector
- Infrared spectrophotometric detector

The flame ionization detector (FID) is the most widely used and generally applicable detector for gas chromatography. Selective detectors such as the electron-capture detector (ECD), flame photometric detector (FPD) and nitrogen-phosphorus detector (NPD) are primarily responsive to specific functional groups. These detectors have found specialized areas of application such as the analysis of environmental samples for pesticides and other pollutants. Spectral detectors such as MS and IR not only serve as sensitive and selective detectors, but also to identify compounds.[1]

For the purposes of this study, the discussion will be limited to detectors that were used to measure the substances under investigation, as described in the literature references, namely the nitrogen-phosphorus detector (NPD) and mass spectrometric (MS) detector. As the principles of the mass separation and ion detection were discussed earlier and apply similarly to GC-MS, the focus here will be limited to ionization for GC-MS.

The nitrogen-phosphorus detector is primarily responsive towards compounds containing nitrogen and phosphorus. The effluent from the gas chromatograph is passed into the detector where a dilute hydrogen and air gas mixture is heated to yield reactive hydrogen atoms. The hydrogen atoms, through a series of chain reactions, produce a highly reactive chemical environment that primarily exists in a gaseous layer near the hot thermionic source. Analyte molecules are decomposed in the hot, chemically active layer and nitrogen or phosphorus

compounds, which form electronegative decomposition products, are selectively ionized. The resulting ion current is then measured at the collector electrode.[2]

Gas chromatography may be coupled to mass spectrometry to provide a powerful tool for quantitative and qualitative analysis of complex sample mixtures. The flow rates employed for capillary columns are generally low enough that the column output may be fed directly into the ionization chamber of the mass spectrometer.

Electron impact ionization may be utilized to ionize solutes eluting from the gas chromatograph in a high vacuum. In electron impact ionization, the analyte molecules are bombarded with electrons in the heated ionization source. The energy of the ionizing electrons is typically regulated to a value of 70 eV by controlling the accelerating voltage, established between the cathode and source housing. When an electron strikes a neutral molecule, it may ionize the molecule by knocking out an additional electron to create a radical cation. In addition to ionization, electron impact ionization may also fragment a molecule to yield a characteristic mixture of ions. Under electron impact conditions the molecular ion is often unstable resulting in the loss of useful information concerning the molecular weight of the analyte.[1,3,12]

Chemical ionization is effected by reacting neutral analyte molecules with a high concentration of reagent gas ions. An electron beam mainly ionizes the reagent gas molecules as these exceed the concentration of the analyte molecules by several orders of magnitude. The source is operated at high pressure compared to electron impact ionization with the result that ion-molecule interactions predominate. Chemical ionization is generally less energetic than electron impact ionization resulting in the production of stable molecular ions or molecular ion adducts with little additional fragmentation. The high pressure environment in the chemical ionization source also favor the production of thermal electrons which

may be captured by molecules with a high electron affinity thus producing negatively charged molecular ions.[3]

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CHAPTER 3

Development and optimization of a sample preparation procedure for the liquid chromatographic analysis of trace levels of 3-alkyl-2-methoxypyrazines in wine

3.1. Introduction

The concentrations of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine are expected to fluctuate between approximately < 0.1 and 14 ng/L.[1] It is essential that the method of analysis utilized is capable of precise measurements in the range of typical values. For the application of compiling a profile of relative abundances of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine, the method should preferably yield detection limits of the order of 0.005 ng/L or better. The ultra-low levels expected for the analytes under investigation necessitate highly sensitive and selective extraction and analysis methods for quantitative purposes. To facilitate the successful liquid chromatography-mass spectrometric (LC-MS) analysis of sub parts-per-trillion levels of 3-alkyl-2-methoxypyrazines in Sauvignon blanc wine, an efficient and compatible sample clean-up and pre-concentration procedure therefore needs to be developed and optimized.

A preliminary investigation revealed that an estimated detection limit in the order of 1 pg of IBMP may be expected from some modern LC-MS systems. It then follows that if the equivalent of 200 mL of wine can be loaded on the column, a limit of detection of 0.005 ng/L of IBMP may be achieved. A sample pre-concentration step is therefore essential in order to achieve the required sensitivity. Analytical liquid chromatographic systems offer injection volumes of up to 1 mL, but large injection volumes may overload the analytical column and

degrade sensitivity and precision, depending on the matrix of the extract. Utilization of large injection volumes (in the order of 1 mL) may therefore be feasible, provided that the extract can be chromatographed without loss of resolution. It is therefore essential that the pre-concentration procedure is selective so that the bulk of the wine matrix components may effectively be eliminated to yield relatively clean, aqueous extracts. The ideal sample preparation procedure should therefore affect pre-concentration of a factor of at least 200 as well as a measure of selectivity for sample clean-up to facilitate large injection volumes, in order to impart the desired sensitivity to the method. The sample preparation procedure should also be robust and impart a relatively small amount of quantitative uncertainty to the method.

The efficiency of solvent extraction, solid phase extraction (SPE) and stir bar sorptive extraction (SBSE) sample preparation techniques were evaluated to determine their suitability for the application. The preliminary experimental work was performed utilizing wine fortified with mg/L quantities of 3-isobutyl-2-methoxypyrazine and measuring the residues with reversed phase liquid chromatography with UV detection. Subsequent experiments, aimed at evaluating the efficiency of the said techniques for the extraction of 3-alkyl-2-methoxypyrazines from the distillates of wine samples, were performed with the LC-MS method utilizing APCI ionization. Where LC-MS was used for measuring the residues, samples were fortified at ng/L levels.

Solvent extraction is a separation method based upon the distribution of a solute between two immiscible liquids. The distribution coefficient of the solute in the two liquids is the equilibrium constant that describes the distribution ratio of the solute between the two phases. Generally, distribution coefficients between two immiscible liquids is sufficiently large to accomplish efficient partitioning of the solute into one or the other liquid.[2]

Solid phase extraction (SPE) is a versatile sample preparation technique utilizing a multitude of adsorbents based on polar, hydrophobic and/or ion exchange interactions.[3] Adsorbent chemistries and parameters for separation are based on the same principles that apply to equivalent liquid chromatographic techniques. Adsorbent material is typically packed in disposable cartridges for convenient application of liquid samples. SPE may be used to establish three important pre-requisites for trace-level analysis namely enrichment, removal of interfering matrix components and changing of the sample 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, the liquid sample may be forced through the conditioned cartridge where the analyte is retained on the phase. The matrix may then be washed off followed by selective elution of the analyte. Alternatively, the conditioned phase may retain interferents while the analyte pass through the column, allowing purification of the sample solution. SPE also offer advantages over conventional liquid-liquid extraction such as enhanced selectivity, speed, broad application range, low solvent consumption and potential automation.

Distillation permits a simple and convenient method for separation of volatile components from a sample mixture. As distillation was also used successfully as part of sample preparation procedures in several publications referenced in this study, the effectiveness thereof was evaluated for sample clean-up and pre-concentration.[4,5,6]

Stir bar sorptive extraction (SBSE) is a new solventless extraction method where the analyte are enriched from the sample matrix by sorption into a polydimethylsiloxane (PDMS)–coated stir bar.[7] Extraction of the analyte may be achieved by stirring directly in liquid samples or alternatively, by placing the bar in the headspace of the agitated or heated sample. Partitioning of the analyte between the PDMS phase and the sample matrix is based on the relevant equilibrium distribution ratio as well as the ratio of phases present. Analysis may

be performed following thermal desorption (for gas chromatographic analysis), or alternatively liquid desorption may be achieved by stirring with a small volume of suitable solvent for subsequent liquid phase separation.[8]

This Chapter reports the evaluation of each of the outlined techniques as well as combinations thereof, for sample preparation in the trace level determination of 3-alkyl-2-methoxypyrazines in wine.

3.2. Experimental

3.2.1. Materials

High purity solvents and chemicals were used throughout the experiment. Dichloromethane (DCM) and tannic acid were from Merck (Darmstadt, Germany), Di-ethyl ether from Saarchem (Merck, South Africa), IBMP, acetonitrile, ethanol and methanol from Aldrich (Sigma-Aldrich, South Africa) hexane from Burdick & Jackson (Honeywell International, U.S.A.), formic acid from Associated Chemical Enterprises (ACE, South Africa) and sodium hydroxide from Sigma (Sigma-Aldrich, South Africa).

Standards were prepared by weighing appropriate amounts of reference material on an analytical balance. Dilutions were made using A-grade volumetric glassware. Intermediate standards were prepared in ethanol while working standards were prepared in 10% ethanol. Wine samples were fortified volumetrically by adding appropriate amounts of standards.

The SPE products that were evaluated were as follows (Table 3.1.):

Table 3.1.: SPE products evaluated in the investigation.

Manufacturer	International Sorbent Technologies Ltd [9]
Description	IST Isolute C18(EC)
Sorbent chemistry	Octadecyl
Sorbent mass	200 mg / 3 mL
Particle size	50 µm
Endcapped	yes

Manufacturer	Waters Corporation [10]
Description	Oasis HLB
Sorbent chemistry	Divinylbenzene and N-vinylpyrrolidone
Sorbent mass	60 mg / 3 mL
Particle size	30 µm

Manufacturer	Waters Corporation [10]
Description	Oasis HLB
Sorbent chemistry	Divinylbenzene and N-vinylpyrrolidone
Sorbent mass	500 mg / 6 mL
Particle size	30 µm

Manufacturer	Phenomenex [11]
Description	Strata SDB-L
Sorbent chemistry	Unmodified polystyrene-divinylbenzene
Sorbent mass	500 mg / 3 mL
Particle size	100 µm

Manufacturer	Waters Corporation [10]
Description	Waters Sep-Pac Vac Silica
Sorbent chemistry	Silica
Sorbent mass	200 mg / 3 mL

Manufacturer	Phenomenex [11]
Description	Strata SCX
Sorbent chemistry	Silica based sulfonic acid
Sorbent mass	500 mg / 3 mL
Particle size	55 µm

3.2.2. Liquid chromatographic methods and instrumentation

An Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) fitted with quaternary pump, autosampler, column oven and UV-visible diode array detector (DAD) was used to analyze the fortified wine samples, distillates and extracts. The liquid chromatographic parameters were as follows: The mobile phase was an acetonitrile/water gradient starting at 50% acetonitrile for 30 seconds, then increasing acetonitrile to 95% in 7 minutes, followed by 95% acetonitrile for 4 minutes, at a flow rate of 0.5 mL/minute. The column was a Phenomenex Luna C18, 150 x 4.6 mm, with 3 µm particle-size, thermostatted at 30°C. Variable injection volumes were used. The chromatographic signal at 292 nm was acquired while spectra between 190 and 400 nm were recorded.

Where the residues were measured with LC-MS, a Waters Alliance LC system (Waters Corporation, Milford, U.S.A.) incorporating a quaternary pump, autosampler, column oven and Micromass Quattro Premier XE tandem quadrupole mass spectrometric detector (Manchester, U.K.), was used. Ionization was achieved utilizing a dedicated APCI probe. The column was a Phenomenex Luna C18 of dimensions 250 x 4.6 mm x 3 µm, thermostatted at 40°C. The mobile phase was a methanol/water gradient, starting at 95% water for 1 minute, then changing to 95% methanol in 18 minutes, followed by 3 minutes at 95% methanol. A variable flow-rate was used, starting at 0.75 mL/minute for 1 minute and then changing with the solvent gradient to 0.85 mL/minute in 18 minutes. Re-equilibration time was 4 minutes, during which the flow also reverted to the starting value of 0.75 mL/minute. Data acquisition was in MRM mode, acquiring data for the relevant primary as well as secondary ion transitions (167 > 125, 167 > 124). The mass spectrometer source parameters were as in Table 3.2.:

Table 3.2.: Source parameters for Ion Sabre APCI probe.

Parameter	Value
Corona needle	4.4 μA
Cone voltage	34 V
Extractor voltage	6 V
RF lens	0 V
Source temperature	150°C
Desolvation temperature	250°C
Cone gas (N_2)	100 L/Hr
Desolvation gas (N_2)	200 L/Hr

3.3. Results and discussion

3.3.1. HPLC method performance

The UV spectrum of IBMP, as measured with the DAD detector, revealed two maxima, at 214 nm and 292 nm, respectively (Figure 3.1.). Despite the fact that the absorption at 214 nm is more intense, the 292 nm wavelength was used for quantitation as less possible interference from the wine matrix components were anticipated at the longer wavelengths.

Using a detection wavelength of 292 nm, the HPLC method provided adequate selectivity for the detection of IBMP, fortified to mg/L levels, in the presence of wine matrix components (Figure 3.2.).

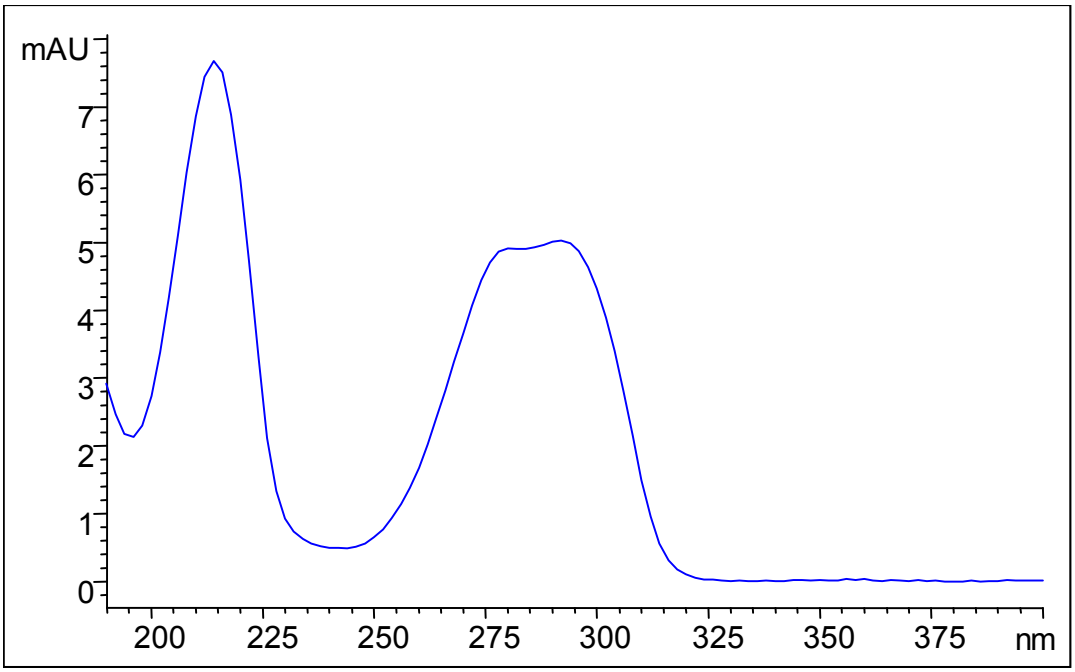

Figure 3.1.: UV spectrum of 3-isobutyl-2-methoxypyrazine, acquired by HPLC-DAD (10 ng in mobile phase).

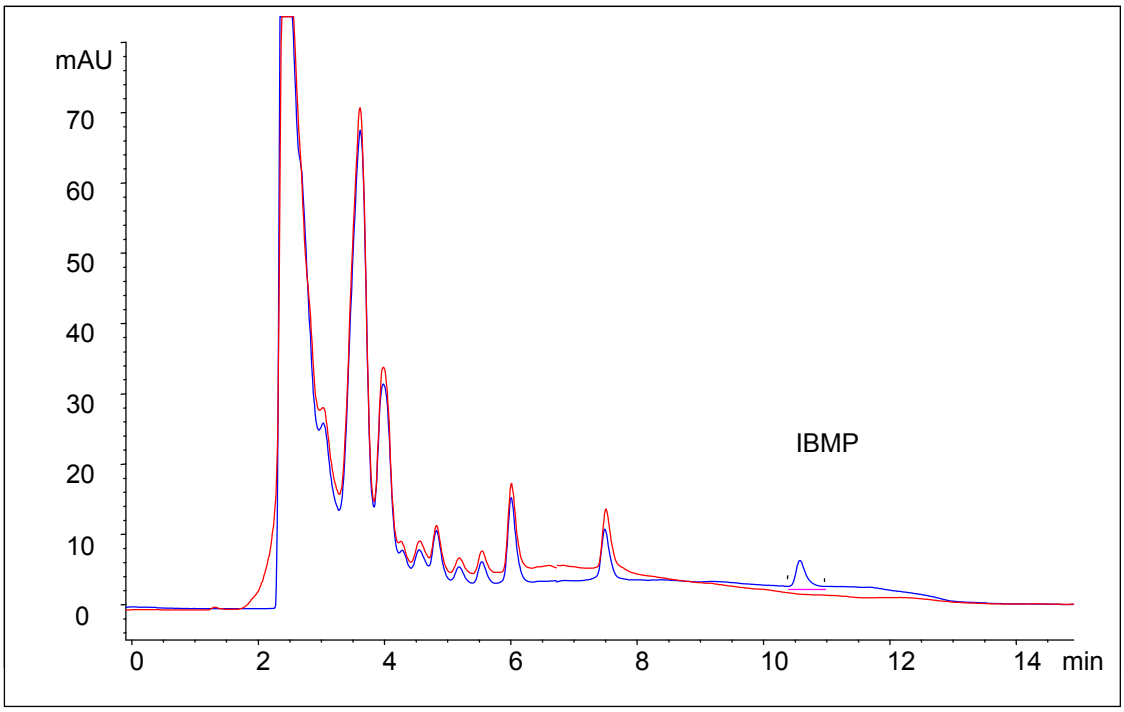

Figure 3.2.: Comparison between chromatograms obtained for a Sauvignon Blanc wine (bottom), and the same wine spiked with 3.0 mg/L of 3-isobutyl-2-methoxypyrazine (top). Injection volume: 10 µL, detection: 292 nm. Other HPLC conditions are as specified in section 3.2.2.

The linear response of the system over the concentration range used during method development was determined by injecting various masses of IBMP. The response of the system was found to be linear over the entire mass range used (Figure 3.3., r = 0.9999). The limit of detection (LOD) was estimated conservatively using a signal-to-noise (S/N) ratio of more than 10, providing a value of 0.50 ng injected on-column. The LOD was determined using a standard solution and therefore does not necessarily apply to real wine extracts.

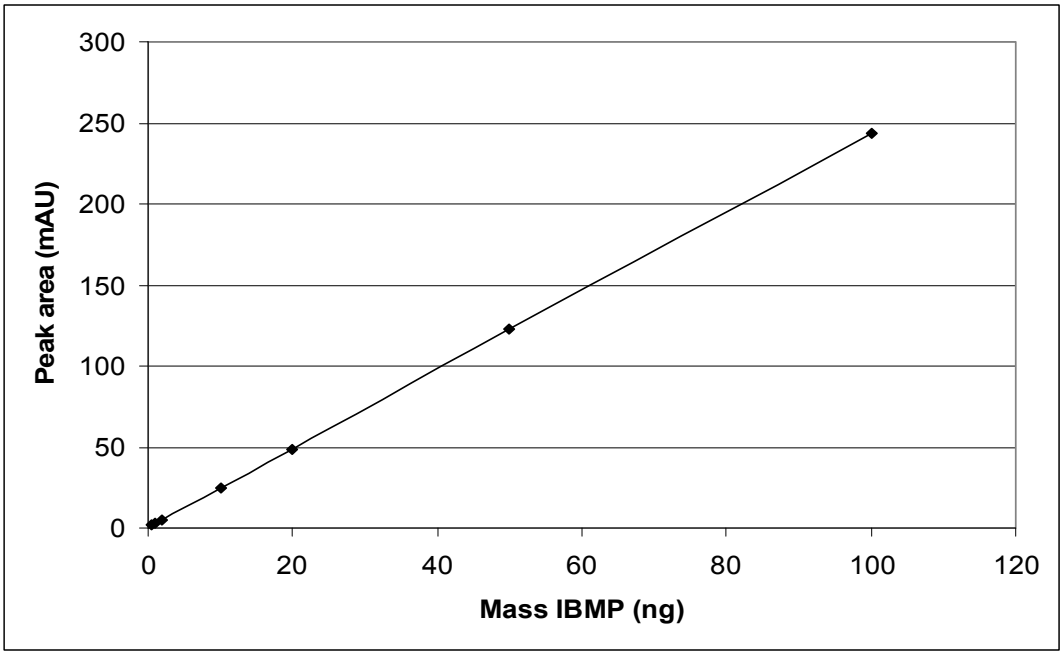

Figure 3.3.: Calibration curve for the HPLC-UV analysis of IBMP standard, 0.50 to 100 ng, r = 0.9999.

The precision of the liquid chromatographic system was evaluated by performing repeated injections of 25 μL of a standard solution containing 2.5 mg/L IBMP. Repeatability, as expressed by the relative standard deviation (RSD) of the peak area, was found to be excellent (0.7% RSD).

3.3.2. Development and optimization of a solvent extraction procedure

3.3.2.1. Comparison of different solvents

It is essential that the analytes of interest display a favorable distribution between the extraction solvent and the sample matrix to ensure their efficient extractive separation from the wine matrix. Several solvents have been used for the extraction of methoxypyrazines from wines. Kotseridis *et al.*[12] used a di-ethyl ether/hexane mixture for the extraction of various compounds including IBMP,

from wine. De Boubee *et al.*[13] used dichloromethane to back-extract IBMP from steam distillates of alkaline wine samples after isolation with ion exchange resin. Dichloromethane, di-ethyl ether and hexane were evaluated in this study.

Table 3.3.: Solvent properties.[14]

Solvent	Boiling point (°C)	Density (20°C) (g/mL)	Vapor pressure (25°C)(kPa)	Dipole moment (D)
Dichloromethane	40	1.3266	58.2	1.60
Di-ethyl ether	34.5	0.7138	71.7	1.15
Hexane	68.73	0.6606	20.2	0

From the information in Table 3.3., it is evident that with relatively low boiling points and high vapor pressures, dichloromethane and di-ethyl ether may potentially be removed readily from sample extracts by evaporation. Removal of the solvent may be essential for successful pre-concentration as well as reversed phase separation of the extracts. Dichloromethane, with relatively high density, may successfully be used for repeated extraction of aqueous solutions utilizing a separating funnel. Di-ethyl ether and hexane on the other hand, are less dense than water and may not conveniently be used to extract aqueous solutions utilizing a separating funnel (without removing the sample solution from the flask with each extraction).

In order to evaluate the efficiency of the various solvents to extract the analyte from the wine matrix, non-equilibrium distribution ratios were determined as a function of time for each solvent. A batch of Sauvignon blanc wine was fortified with 5.0 mg/L of IBMP. Aliquots of 200 mL were then extracted with 10 mL of each of the solvents under investigation. In the case of di-ethyl ether, 15 mL was used because of the relatively high solubility of this solvent in the wine matrix. All mixtures were stirred at a constant rate and sub-samples removed at fixed time intervals. A slow stirring speed was maintained as excessive agitation of the mixture led to the formation of an unresolved gelatinous phase. The results are summarized in Figure 3.4.

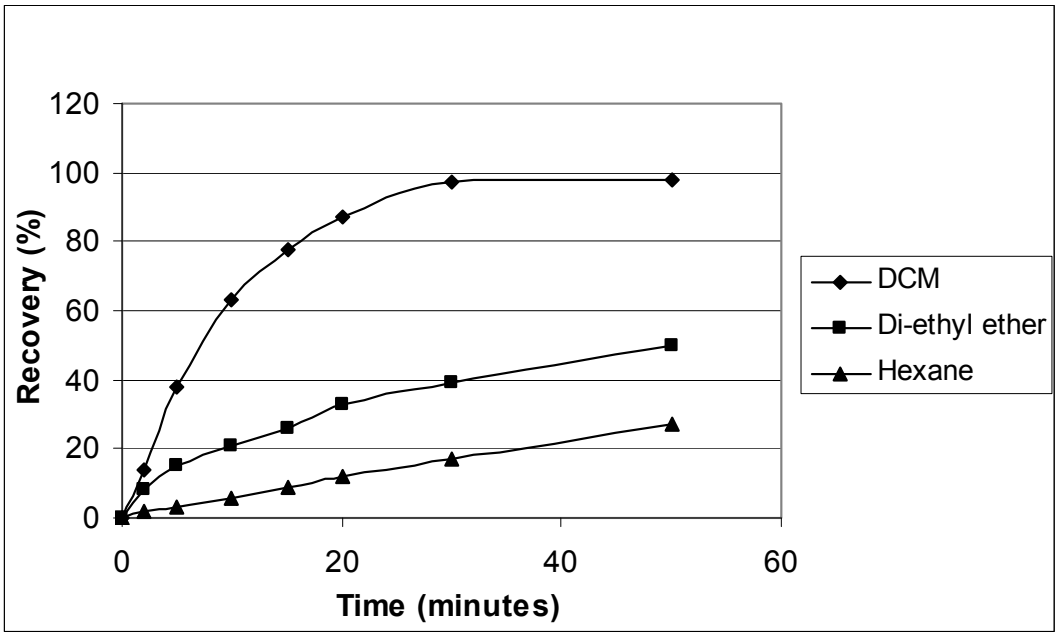

Figure 3.4.: Recovery of IBMP from a spiked wine sample (5.0 mg/L) for dichloromethane, di-ethyl ether and hexane as a function of extraction time.

It is evident from Figure 3.4. that dichloromethane is the best-suited extraction solvent for this application. In addition to higher recoveries for dichloromethane, this solvent is also denser than wine, which facilitates successive extractions with small aliquots of solvent when utilizing a separating funnel. This step is vital for the efficient extraction of IBMP. Dichloromethane also possesses a relatively high vapor pressure (58.2 kPa at 25ºC [14]), which suggest that the extracts may be evaporated readily for concentration purposes.

3.3.2.2. Optimization of extraction parameters using dichloromethane

The non-equilibrium distribution ratios of IBMP between the wine matrix and dichloromethane were used to provide guidance as to an efficient the extractive separation procedure using this solvent. The factors that were evaluated include

the number of extractions, the amount of solvent used in each extraction and the equilibration time for each extraction.

It was demonstrated that at least 87% of IBMP was extracted from 200 mL of wine in 20 minutes with 10 mL solvent (Figure 3.4.). Consequently, the number of additional extractions that are required to extract the remaining IBMP (using a constant extraction time of 20 minutes) were determined. A batch of Sauvignon blanc wine fortified with 5.0 mg/L of IBMP was once again used in these experiments. An aliquot of 200 mL of the fortified wine was extracted with two 10 mL aliquots of dichloromethane for 20 minutes followed by three further 20 minute extractions with 5 mL of solvent. Small volumes of wine were removed for analysis after each extraction to determine the amounts of IBMP remaining. The results are summarized in Figure 3.5. From the data it is evident that three extraction steps are optimal as negligible amounts of IBMP remains in the wine after that.

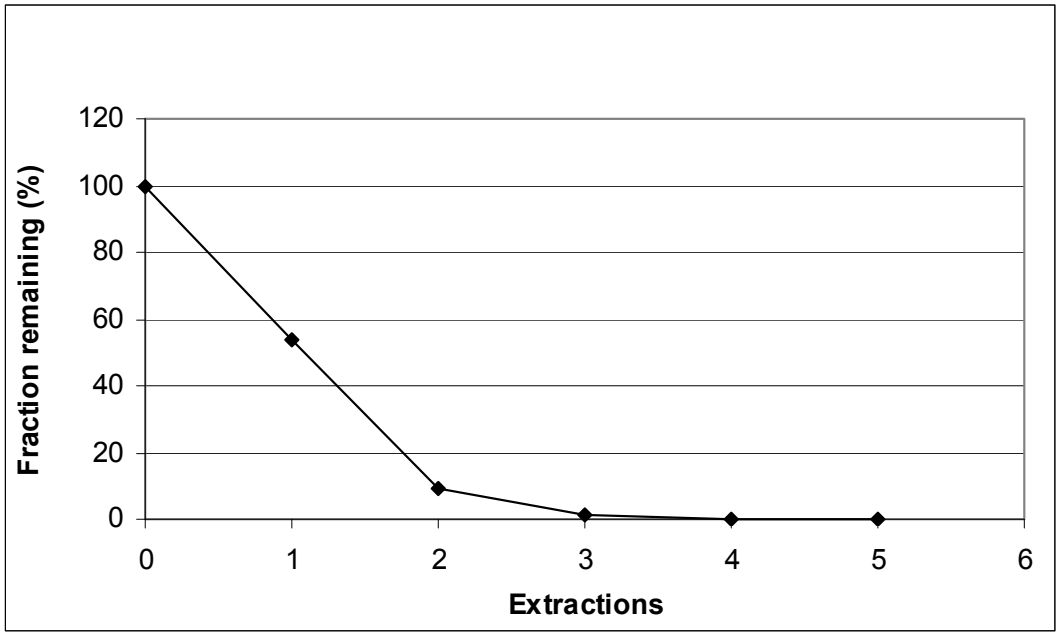

Figure 3.5.: Fraction of IBMP remaining in wine as a function of number of extractions with dichloromethane.

Following multiple extractions, the solvent was evaporated prior to reconstitution in the HPLC mobile phase as at the start of the gradient (50% acetonitrile). In order to avoid analyte loss, concentrated formic acid (0.5 mL) was added to the extracts prior to the evaporation step. The acidic medium ensures that the pyrazines are protonated and therefore reduces loss due to evaporation.

The optimized solvent extraction procedure is summarized below:

- Using an A-grade volumetric flask (200 mL), accurately transfer 200 mL of wine to a 250 mL separating funnel containing a 35 mm egg-shaped magnet.
- Add 10 mL of dichloromethane to the separating funnel.
- Stopper tightly and stir for 20 minutes at slow speed.
- Allow phases to separate and drain the organic phase into a graduated pear-shaped flask containing 0.5 mL concentrated formic acid.
- Mix the contents of the flask on a vortex mixer and place under gentle stream of nitrogen.
- Repeat with 10 mL followed by 5 mL portions of dichloromethane.
- Evaporate the dichloromethane from the extract until less than 0.5 mL remains.
- Reconstitute to 1 mL with mobile phase, vortex and transfer to a 1.5 mL vial for injection.

The repeatability of the optimized procedure was assessed by seven recovery experiments using a Sauvignon blanc wine fortified with 2.5 mg/L of IBMP. Recovery was determined by comparison of the peak area of IBMP in the fortified wine prior to extraction and in the reconstituted extracts. The average of seven recovery measurements was 95%, with an RSD value of 11%.

It was demonstrated that dichloromethane could be utilized for the solvent extraction of mg/L quantities of IBMP from wine. The solvent extraction

procedure described is capable of practically quantitative extraction of IBMP from Sauvignon blanc wine with good repeatability and a concentration factor of 200. From a practical perspective, it was found that it is essential to stir the mixture slowly with a large magnet to ensure efficient extraction while preventing the formation of emulsions. An example of an HPLC chromatogram obtained for the extraction of a Sauvignon Blanc wine fortified with 2.5 mg/L IBMP is presented in Figure 3.6.

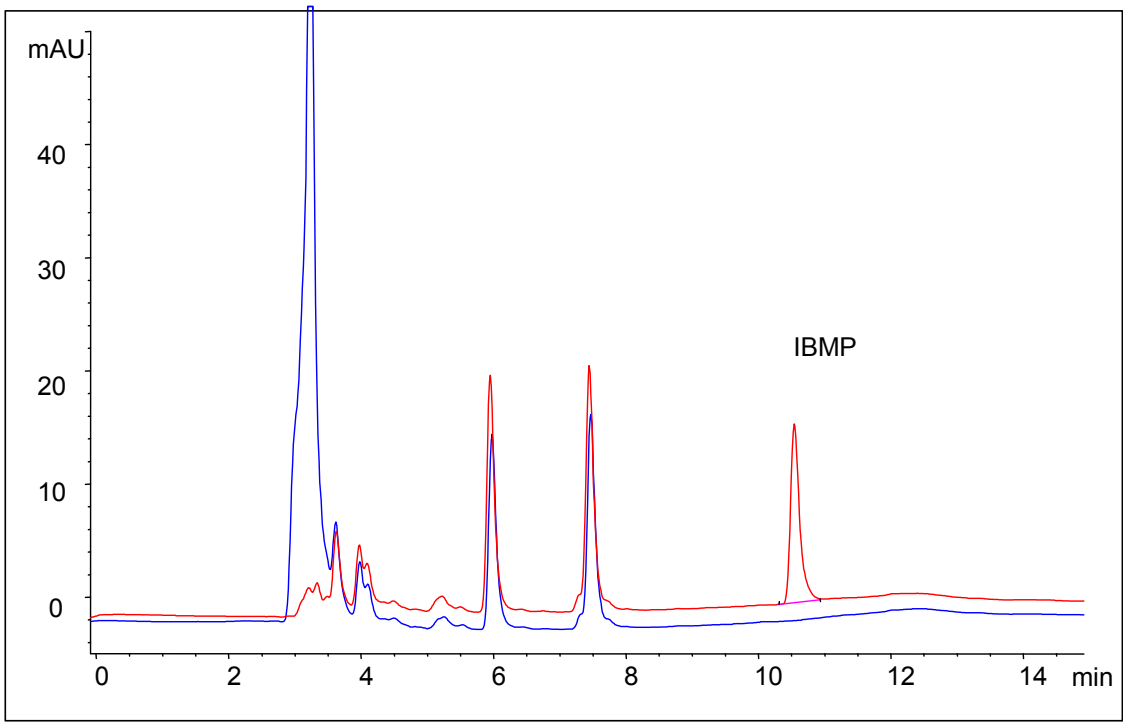

Figure 3.6.: Chromatograms obtained for an extract of Sauvignon blanc wine containing no measurable quantities of IBMP (bottom) and the same wine fortified with 2.5 mg/L IBMP (top). Concentration factor 200, 0.2 µL injected.

3.3.3. Evaluation of distillation as sample treatment for the analysis of methoxypyrazines in wine

The dichloromethane extraction procedure described above suffers from relatively poor selectivity, with the result that many components of the wine matrix are co-extracted with the analytes of interest. Due to the high concentration factor of the extraction procedure, wine matrix components are expected to be present in high concentrations. Matrix interference may therefore be expected to limit the HPLC injection volume, as overload of the column will result in a loss of chromatographic resolution. Resolution of the analyte is an important parameter as a loss in sensitivity will result from poor resolution. Very high concentrations of co-eluting matrix elements may also possibly interfere with efficient mass spectrometric detection of the components of interest.

The 3-alkyl-2-methoxypyrazines are known to be volatile and several authors reported the use of distillation as part of successful sample preparation procedures for the determination of IBMP in wine.[13,15] Distillation may therefore be used in combination with solvent extraction to improve the efficiency of the sample preparation procedure. By separating the 3-alkyl-2-methoxypyrazines from the non-volatile components of the wine matrix, distillation may impart an extra measure of selectivity, thus reducing the matrix of the extract. Distillation will also serve to reduce the volume of sample to be extracted as well as the amount of solvent required for the extraction. This will provide the added advantage of reducing the time required for evaporation of the solvent. In addition, the efficiency of the sample preparation procedure may also be improved as larger effective volumes of sample may be extracted resulting in increased concentration factors while reducing the required equilibration time.

The extent of wine matrix co-extraction as well as potential selectivity benefits of distillation was estimated by recording the chromatographic signal at 195 nm of a dichloromethane extract of a wine and the extract of a distillate of the same wine.

An aliquot of 200 mL of wine was extracted with dichloromethane as described previously. The same volume of the wine was then distilled followed by dichloromethane extraction of the distillate. Equal volumes of the two extracts thus obtained were then analyzed to determine the comparative reduction in matrix components brought about by distillation.

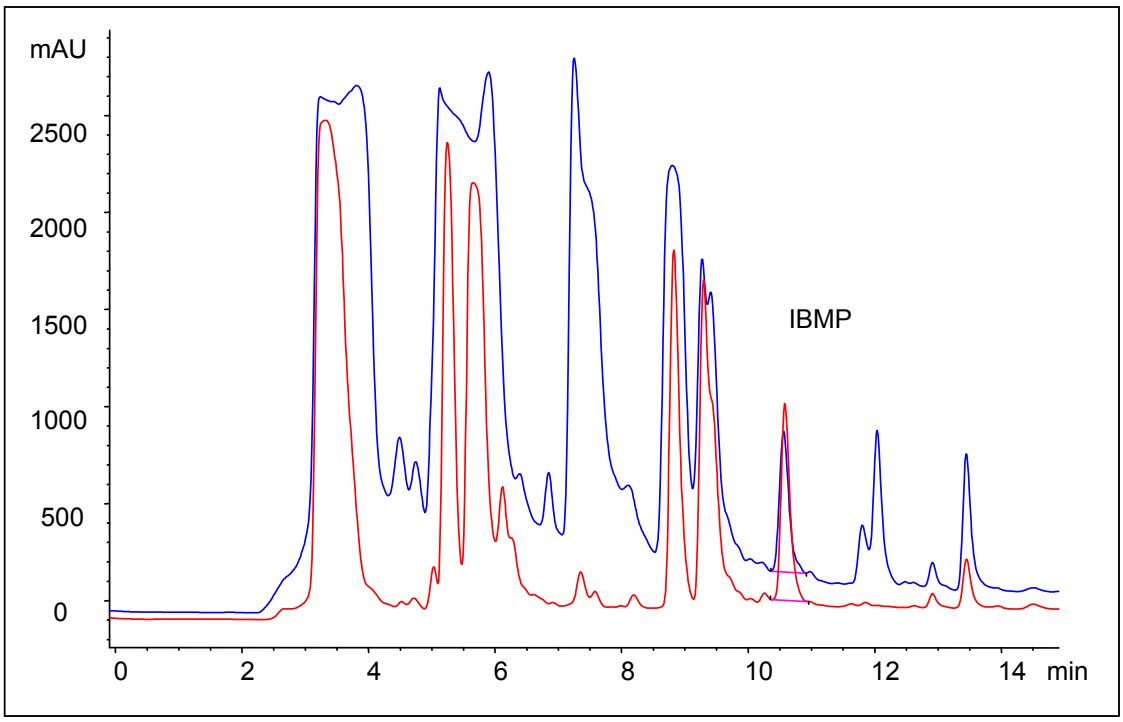

Figure 3.7.: Overlay of chromatograms of an extract of Sauvignon blanc wine (top), and the extract of the distillate of the same wine (bottom). Detection: 195 nm. Concentration factor 200, 25 µL injected.

From Figure 3.7. it is clear that distillation of the wine prior to extraction eliminates many matrix components from the extract. It should be noted that Figure 3.7. does not reveal all components that may possibly be eliminated by distillation. Sugars, which are a major matrix component, would also be eliminated by distillation but as the sugars do not absorb UV light, the effect of the elimination of such compounds from the extract is not reflected in Figure 3.7.

3.3.3.1 Optimization of distillation parameters for the extraction of methoxypyrazines from wine

A premise for the successful utilization of distillation to improve the efficiency of the sample preparation procedure is quantitative recovery of the analyte from the sample by the distillation process.

The 3-alkyl-2-methoxypyrazines are organic bases, which may potentially be protonated at low pH to form non-volatile quaternary ammonium ions. Hartman *et al.* reported that the concentration of the alkyl-methoxypyrazines in the headspace of spiked model wines were significantly reduced at a matrix pH below 2, and was virtually eliminated below pH 1. In contrast, recovery values remained fairly constant between pH 3 and 11.[16] Wine generally has a pH of between 3 and 4 and adjustment of the pH to improve recovery should therefore not be required.[17] The effect of sample pH on the recovery of IBMP during distillation was nevertheless evaluated. In addition, the optimal volume of distillate that needs to be collected from a particular volume of sample distilled was also determined.

In order to evaluate the efficiency of distillation to recover the analyte from the wine matrix, fortified samples were distilled into different fractions in order to determine the amount of IBMP in each fraction. A batch of Sauvignon blanc wine that was fortified with 2.0 mg/L IBMP was used in these experiments. Aliquots of 200 mL of the fortified wine were distilled utilizing simple distillation and the distillate collected in 25 mL fractions. To investigate the effect of pH on the volatility of the analyte, the pH of two wine samples were adjusted with sodium hydroxide. Recoveries were determined by analyzing the various fractions thus obtained. The results of these experiments are summarized in Figure 3.8.

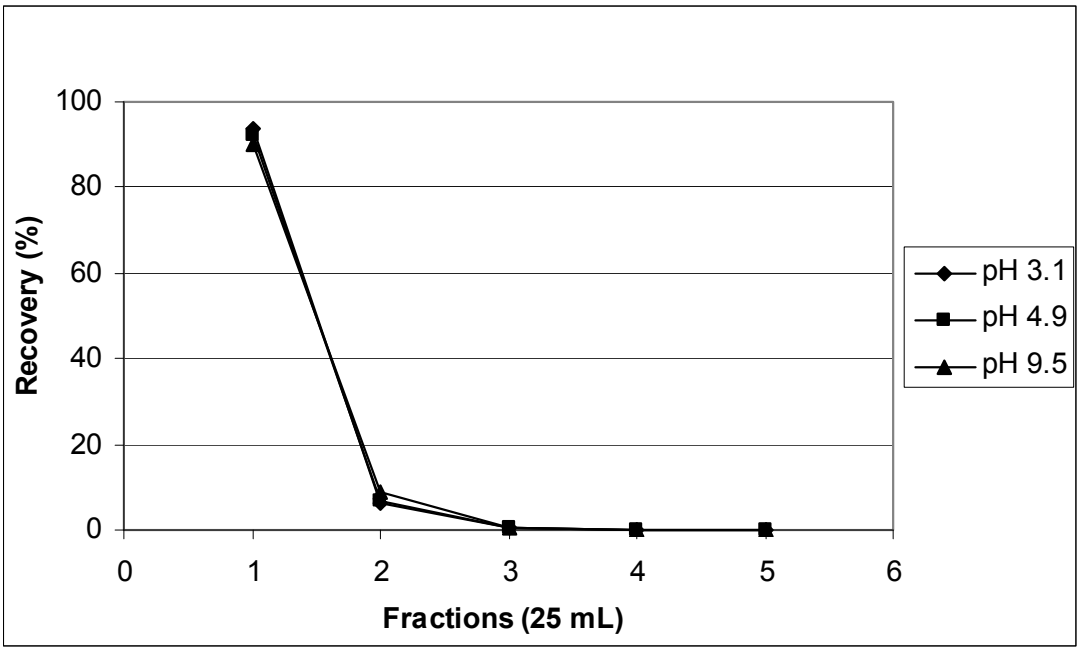

Figure 3.8.: Recovery of IBMP from 200 mL wine at various pH values by distillation.

From the data it can be observed that IBMP is practically quantitavely recovered from 200 mL wine in the first three 25 mL fractions. It should therefore be sufficient to distil the sample to a volume of equivalent to 50% of the original sample volume where simple distillation is used. It was also demonstrated that wine pH, in the range 3.1 to 9.5, does not affect the recovery of IBMP from wine by distillation.

The possibility that fractional distillation will provide more efficient separation of the analyte from less volatile components of the wine matrix was also investigated. A batch of Sauvignon blanc wine was fortified with 2.0 mg/L IBMP and aliquots of 200 mL and 500 mL were distilled utilizing a 60 cm fractionating column while the distillate was collected in 25 mL fractions for analysis. The results are summarized in Figure 3.9.

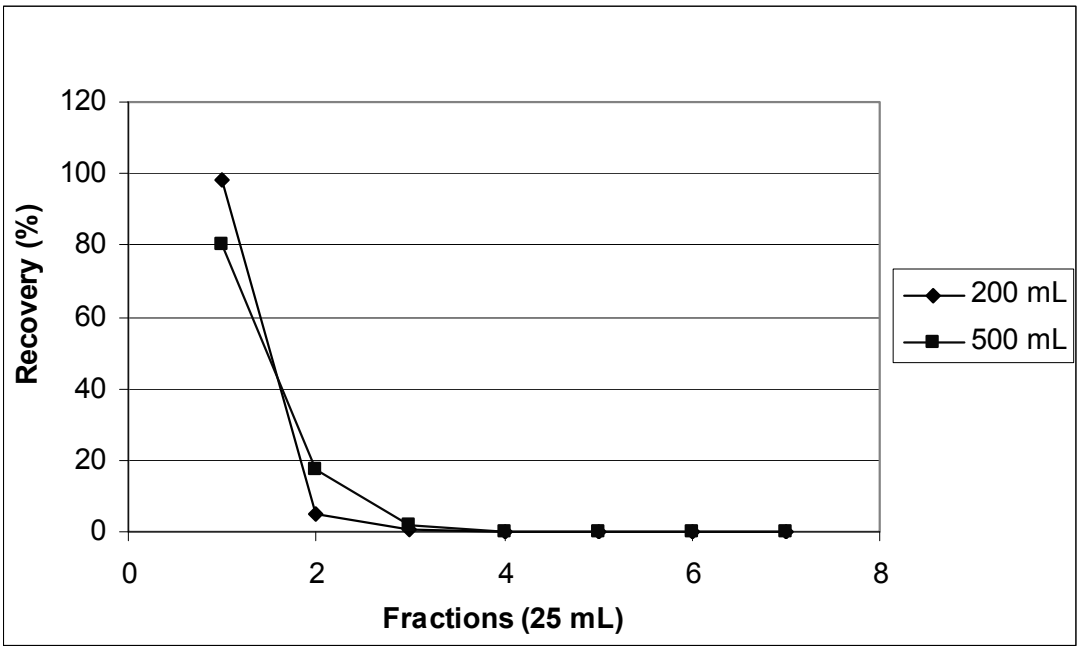

Figure 3.9.: Recovery of IBMP from 200 mL and 500 mL of wine by fractional distillation utilizing a 60 cm column.

Practically all the IBMP is recovered in the first two and three 25 mL fractions for sample volumes of 200 and 500 mL, respectively. It should therefore be sufficient to distil the sample to a volume of equivalent to 25% of the original sample volume when fractional distillation (60 cm column) is used.

The efficiency of different distillation procedures was evaluated by comparing simple distillation to fractional distillation utilizing columns of 40 cm, 60 cm and 100 cm. Efficiency was evaluated by determining the smallest volume of distillate that quantitatively contains the analyte as well as the extent of matrix co-extraction in this fraction. Aliquots of 500 mL of wine fortified with 2.0 mg/L IBMP were distilled while the distillates were collected in 25 mL fractions. The results are summarized in Table 3.4.

Table 3.4.: Recovery of IBMP from wine obtained by different distillation procedures.

Fractions (25 mL)	Simple Distillation (%)	40 cm Column (%)	60 cm Column (%)	100 cm Column (%)
1	68.0	73.0	80.5	77.8
2	25.1	22.7	17.3	17.4
3	5.3	4.2	2.0	2.8
4	1.0	0.1	0.1	0.9
5	0.3	< 0.1	< 0.1	0.4
6	0.1	< 0.1	< 0.1	0.3
7	0.1	< 0.1	< 0.1	0.3
8	0.1			

As is evident from Table 3.4., the 60 cm fractionating column provided the best recoveries. It is also evident that the analyte is practically quantitatively contained in 100 mL of the distillate thus obtained. Chromatograms recorded at 192 nm to compare the extent of matrix co-extraction did not reveal significant differences between the amounts of matrix components in each of the distillates (results not shown).

3.3.3.2. Optimization of dichloromethane extraction of IBMP from wine distillate

In order to optimize the dichloromethane extraction of the distillate, recoveries of IBMP were determined as a function of extraction time. A Sauvignon blanc wine was fortified to contain 2.0 mg/L of IBMP upon which aliquots of 500 mL of the fortified wine was distilled to 250 mL. The distillate was then extracted with 10 mL and 5 mL aliquots of dichloromethane, respectively while sub-samples were removed at fixed time intervals. The results are presented in Table 3.5.

Table 3.5.: Recovery of IBMP from the distillates of a fortified wine sample. Extractions were with 5 and 10 mL of dichloromethane respectively.

Time (Min.)	Recovery (%) 10 mL Dichloromethane	Time (Min.)	Recovery (%) 5 mL Dichloromethane
0	0	**0**	0
10	96	**2**	53
20	96	**5**	63
30	95	**10**	69
40	95	**20**	72
50	95	**30**	71
60	95	**40**	70

Non-equilibrium distribution ratios for IBMP between the distillate and dichloromethane were used to provide guidance as to an efficient extraction procedure utilizing dichloromethane. The number of extractions, amount of solvent used in each extraction and the equilibration time for each extraction were subsequently optimized.

At least 95% of IBMP was extracted from the distillate (250 mL) of 500 mL wine in 10 minutes with 10 mL dichloromethane. This value drops to 70% in the same time when 5 mL dichloromethane is used instead. This can be explained by corresponding changes in the ratios of extraction solvent to sample matrix. An aliquot of 10 mL dichloromethane and 10 minutes extraction time were therefore selected as optimal for the first extraction step. As relatively small amounts of IBMP are left after this, extractions with 5 mL for 10 minutes are performed thereafter. Using this methodology, the recovery of IBMP from the distillate of a fortified wine (2.0 mg/L) is summarized in Figure 3.10. It is evident that utilizing this procedure, three extraction steps are sufficient to quantitatively extract IBMP from the distillate.

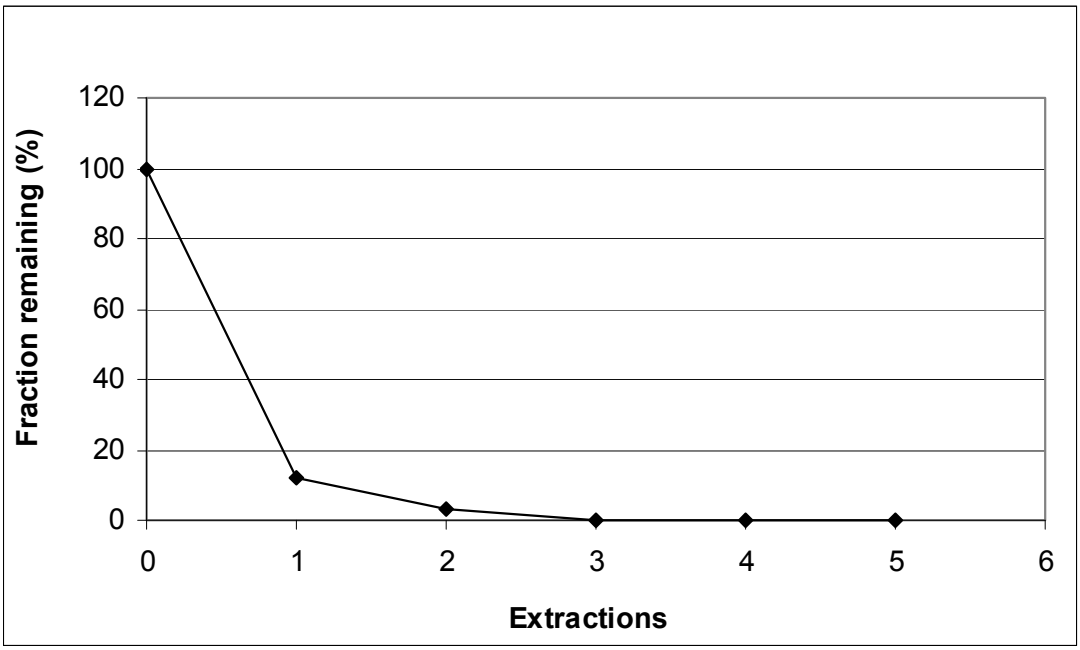

Figure 3.10.: Fraction of IBMP remaining in the distillate (250 mL) of 500 mL wine upon extraction with dichloromethane.

The optimized sample preparation procedure utilizing distillation followed by dichloromethane extraction is summarized below:

- Using an A-grade volumetric flask (500 mL), accurately transfer 500 mL of wine to a 1 L boiling flask containing a single glass ball. To prevent the wine from foaming, a spatula tip of tannic acid may be added.
- Distill the wine until a volume of at least 100 mL is collected. The distillate is collected in a 100 mL volumetric flask.
- Transfer the distillate quantitatively (rinsing with water) to a 500 mL separating funnel and add 10 mL of dichloromethane as well as a 35 mm egg-shaped magnet.
- Stopper tightly and stir for 10 minutes at high speed.
- Allow phases to separate and drain the organic phase into a graduated pear-shaped flask containing 0.5 mL concentrated formic acid.
- Mix the contents of the flask on a vortex mixer and place under gentle stream of nitrogen.

- Repeat with two 5 mL portions of dichloromethane.
- Evaporate the combined dichloromethane fractions from the extract until less than 0.5 mL of formic acid remains.
- Reconstitute to 1 mL with mobile phase, vortex and transfer to a 1.5 mL vial.

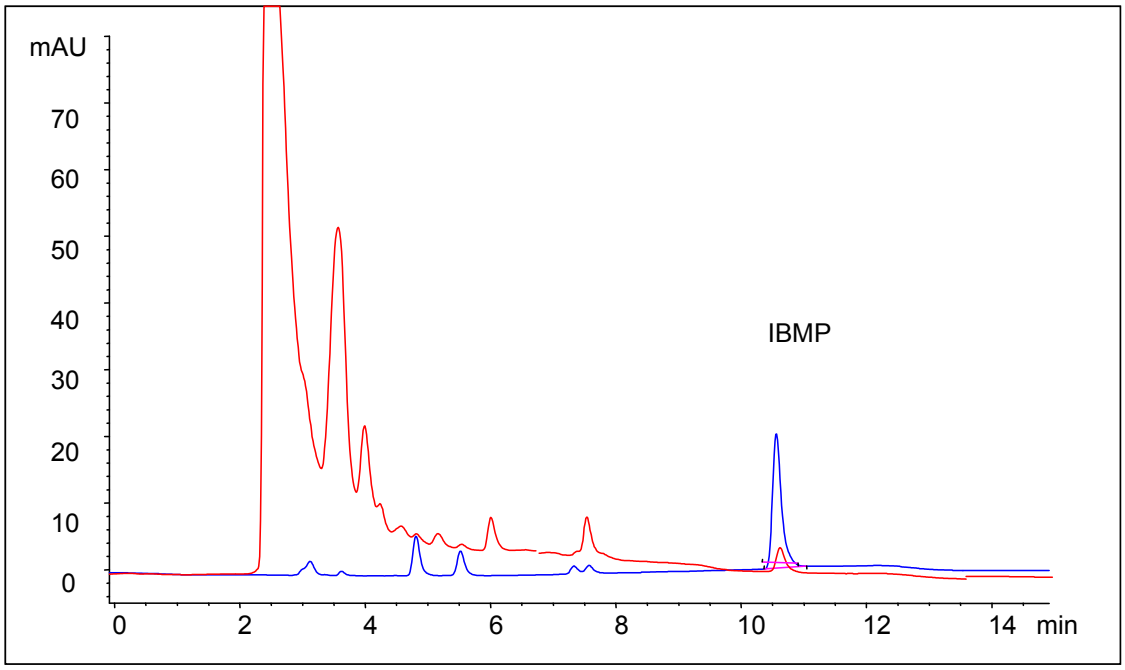

Figure 3.11.: Comparison between chromatograms obtained for a Sauvignon Blanc wine fortified with 2.0 mg/L IBMP (25 µL injected, top), and the dichloromethane extract of the distillate of the same wine (bottom). Concentration factor 500, 0.1 µL injected. Other HPLC conditions are as specified in section 3.2.2.

The effectiveness of the optimized distillation/extraction procedure was assessed by the recovery of IBMP from wine using seven extractions of a Sauvignon blanc wine (fortified with 2.0 mg/L IBMP). Recovery was determined by comparison of the peak areas obtained for the fortified wine, distillate and reconstituted extracts, respectively. The recovery for the distillation process was found to be 102% (RSD = 0.6%), while the total recovery was 110% (RSD = 12%).

In conclusion, it was demonstrated that distillation in combination with dichloromethane extraction offers a very efficient sample pre-treatment step for the recovery of IBMP from wine while yielding an effective concentration factor of 500. Figure 3.11. shows chromatograms of a fortified wine and the corresponding extract.

3.3.4. Evaluation of solid phase extraction for the isolation and pre-concentration of 3-isobutyl-2-methoxypyrazine from wine

As the IBMP molecule posses apolar functionality in addition to the relatively polar methoxy- and aromatic amine functionalities, normal phase, reversed phase as well as ion exchange SPE chemistries were evaluated for the SPE study. Each separation was optimized to determine the capacity of the relevant SPE cartridge as well as optimal conditions for elution of interferences. Recovery and the level of enrichment of the analyte from the wine matrix were also determined in each case.

3.3.4.1 SPE on reversed phase C18 sorbents

In reversed phase chromatography, the stationary phase is apolar and the mobile phase is a relatively polar solvent.[2] The principles that were discussed for reversed phase liquid chromatography is equally applicable to reversed phase SPE.

IBMP is expected to be retained strongly via reversed phase mechanism on an apolar C18 phase as the molecule posses a five-carbon alkyl chain. The column (200 mg / 3 mL) was conditioned by applying 1 mL of acetonitrile followed by 1 mL of water. Aliquots of 10 mL of wine, fortified to 2.0 mg/L, were applied to the

column and the effluent collected in separate fractions for HPLC-UV analysis. The capacity of the column was determined by analyzing each effluent fraction and determining the sample volume corresponding to analyte breakthrough. Breakthrough for IBMP was observed following application of more than 80 mL of wine. It was therefore assumed that 50 mL is the optimal sample volume (Figure 3.12.).

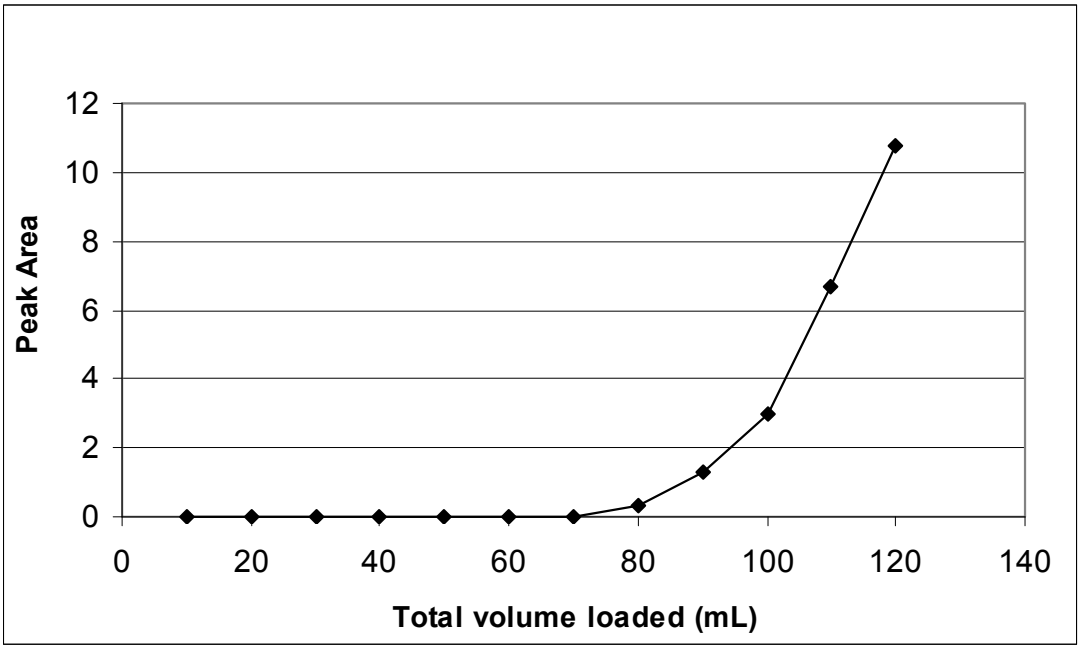

Figure 3.12.: IBMP breakthrough on C18 SPE column.

Optimal conditions for removal of the sample matrix were then determined by loading 10 mL of fortified wine onto several columns and applying 1 mL of a wash solution containing increasing amounts of acetonitrile to each column. The effluents were collected separately for liquid chromatographic analysis to determine the fractions that contained eluted analyte. Wash solutions of pure water and 20, 40, 60 and 80% acetonitrile were evaluated. Breakthrough of IBMP was observed for wash solutions containing 40% or more of acetonitrile. A solution of 20% acetonitrile was therefore selected as the most efficient wash solvent under the described conditions.

The efficiency of the optimized C18 SPE process was evaluated by determining the average recovery of IBMP from wine. An aliquot of 50 mL of fortified wine (2.0 mg/L) was loaded onto a conditioned column followed by rinsing with 1 mL of 20% acetonitrile in water. The analyte was then eluted from the column with three 1 mL portions of acetonitrile and the eluent transferred to a 50 mL pear-shaped vessel with a 3 mL graduated stem. To prevent volatilization of the analyte during reduction of the solvent, 0.5 mL concentrated formic acid was added to the solution. The solution was then evaporated to approximately 0.5 mL and made up to 1 mL with water, thus producing a concentration factor of 50. This procedure was repeated three times, and the average recovery of IBMP was measured as 88% (6.1% RSD, n = 3).

3.3.4.2. SPE on mixed mode reversed phase cartridges

3.3.4.2.1. Direct extraction from wine

New sorbent types for SPE that are designed to have a hydrophilic-lipophilic balance when used under reversed phase conditions, have recently become available, and are designed to enhance retention of acidic, basic as well as neutral compounds. One such phase, marketed under the name Oasis, contains a sorbent consisting of a copolymer of divinylbenzene and N-vinylpyrrolidone units. The divinylbenzene units impart lipophilic character to the phase while the N-vinylpyrrolidone units provide hydrophilic interactions.[10]

It is expected that IBMP will be strongly retained on the Oasis phase as the molecule posses an apolar alkyl chain as well as relatively polar aromatic amine- and methoxy functional groups.

The 60 mg / 3 mL Oasis column was conditioned, as prescribed, by applying 1 mL of methanol followed by 1 mL of water. Aliquots of 10 mL of fortified wine were then applied to the column and the effluent collected separately. The capacity of the column was determined by analyzing the fractions to determine the sample volume corresponding to analyte breakthrough. The results indicated that breakthrough occurred upon loading sample volumes of larger than 100 mL (Figure 3.13.). It was therefore assumed that 50 mL is the optimal volume of wine that can be extracted under the described conditions if breakthrough is to be avoided.

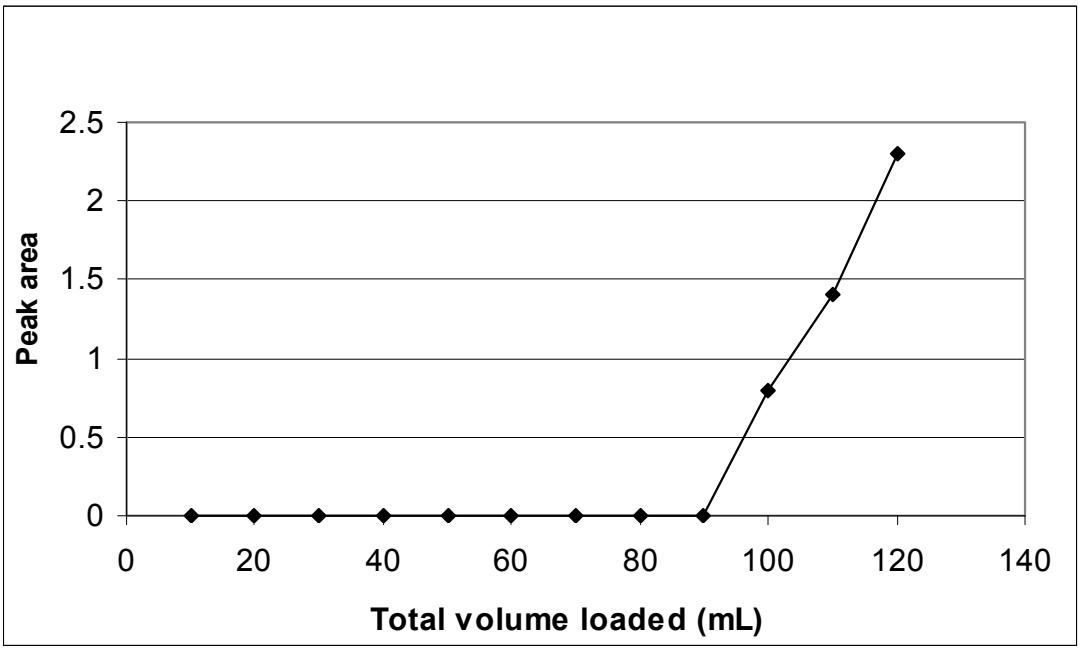

Figure 3.13.: IBMP breakthrough on Oasis column, direct extraction of wine.

Optimal conditions for removal of the sample matrix were determined by loading 10 mL of fortified wine onto several conditioned columns and applying wash solutions to the column as prescribed by the manufacturer. An initial wash with 1 mL of 5% aqueous methanol was applied to all columns, followed by rinsing with 1 mL of solutions containing 2% ammonia and increasing amounts of methanol in

water (10, 20, 30, 40 and 50% methanol were evaluated). A basic rinsing solution is used to ensure that the analyte remain neutral (and therefore relatively apolar), thus maximizing retention. Effluents were collected separately for liquid chromatographic analysis to determine the fractions that contained eluted analyte. It was found that only a solution of 2% ammonia and 50% methanol in water, applied after the initial wash, eluted some solute from the column. A solution of 2% ammonia in 30% methanol was therefore considered to be the most efficient wash solvent under the described conditions.

The efficiency of the process was evaluated by determining the recovery of IBMP from wine. An aliquot of 50 mL of fortified wine (2.0 mg/L) was loaded onto a conditioned column. Elution of matrix components took place in a two-step process, which consisted of applying first 1 mL of a solution of 5% methanol in water followed by 1 mL of 2% ammonia and 30% methanol in water. The analyte was then eluted from the column with three 1 mL aliquots of a solution of 2% acetic acid in methanol. Protonation of the analyte by the acetic acid renders it relatively polar to enhance elution from the column. The solvent was then evaporated and reconstituted as above (for C18 SPE) to produce a concentration factor of 50. The average recovery for this procedure was 79% (5.1% RSD, n = 3).

3.3.4.2.2. Extraction from the distillate of a wine sample

Distillation of wine samples, prior to solvent extraction, provided an effective method for elimination of non-volatile matrix components. Subsequent separation of extracts thus obtained on a C18 column followed by LC-APCI-MS analysis revealed that some unknown matrix components persisted, which interfered with the determination of IPMP. As Oasis columns provided the best performance regarding sample clean-up and pre-concentration, of the SPE products that were

evaluated, the use thereof in tandem with distillation was evaluated to eliminate the said interferon.

The 500 mg / 6 mL Oasis column was conditioned by applying 5 mL of methanol followed by 5 mL of water. An aliquot of 500 mL wine (fortified to 200 ng/L) was then distilled utilizing a 60 cm fractionating column and the first 100 mL of the distillate collected. The distillate was transferred to a 500 mL separating funnel and extracted with dichloromethane as described in section 3.3.3.2. The combined extracts were reduced to approximately 2 mL and subsequently reconstituted in 25 mL 2% ammonia in water for application to the SPE column. The capacity of the column was determined by analyzing the fraction corresponding to the last 2 mL of solution that was passed through the column to ensure complete analyte retention. For these experiments the content of methoxypyrazines were measured with LC-APCI-MS as outlined in the experimental section.

Removal of the sample matrix was optimized by applying 1 mL aliquots of rinsing solvents consisting of 2% ammonia in water and 10 to 90% methanol (in 10% increments) to the column. Each effluent was collected separately for LC-MS analysis to determine the fractions that contained eluted analyte.

It was found that a solution of 2% ammonia in 90% methanol eluted measurable quantities of the analyte from the column. A solution of 2% ammonia and 70% methanol in water was therefore considered to be the most efficient wash solvent. It was subsequently confirmed that elution with three 1 mL aliquots of this (latter) solution did not remove measurable quantities of the 3-alkyl-2-methoxypyrazines from the column. Elution of the analyte was achieved utilizing a solution of 5% formic acid in acetone.

Repeatability of the optimized procedure was evaluated by determining the recovery of IBMP from a wine sample, fortified to contain 200 ng/L IBMP.

Aliquots of 500 mL wine were distilled and 100 mL of the distillate collected. Distillates were extracted with dichloromethane and the combined fractions reduced under nitrogen, at room temperature, to approximately 2 mL. This solution was then reconstituted to 25 mL with a solution of 2% ammonia in water and applied to a pre-conditioned SPE cartridge. The collection vessel was rinsed three times with 1 mL of water and transferred to the cartridge, prior to washing with three 1 mL aliquots of 2% ammonia in 70% methanol. Analytes were eluted from the column with five 2 mL aliquots of a solution of 5% formic acid in acetone. The solvent was evaporated and the solution reconstituted as before, thus producing a concentration factor of 500. Residual formic acid served to protonate the analyte, thus rendering it less volatile. Residues were measured with LC-APCI-MS. The average recovery of IBMP from the wine matrix was 82% (RSD = 4.3%, n = 3).

3.3.4.3. Reversed phase SPE on polystyrene-divinylbenzene (PSDVB) cartridges

The polystyrene-divinylbenzene phase is a highly cross-linked polymer that is unmodified and does not exhibit secondary interactions.[11] The phase is suited for non-selective retention of solutes of low to moderate polarity via a reversed phase mechanism. The large surface area associated with the polymeric sorbents imparts a relatively high capacity to the phase.

A PSDVB cartridge was conditioned with 1 mL of acetonitrile followed by 1 mL of water. Aliquots of 10 mL of wine, fortified with 2.0 mg/L IBMP, were applied consecutively and each effluent fraction collected separately for HPLC-UV analysis. After a total volume of 120 mL was loaded without saturation of the column, the aliquots were increased to 30 mL. A total volume of 570 mL wine was eventually loaded onto the column without saturation of the phase. It was therefore assumed that the column has a capacity of at least 500 mL.

Elimination of the sample matrix was optimized by loading 10 mL aliquots of fortified wine onto several columns and applying 1 mL of wash solutions containing increasing amounts of acetonitrile (20, 40, 60 and 80% in water) to each column. Each effluent was collected separately for HPLC-UV analysis to determine the fractions that contained eluted analyte. Since small amounts of analyte were detected in the 40% acetonitrile fraction, a solution of 20% acetonitrile was selected as the most efficient wash solvent.

The efficiency was evaluated by determining the average recovery from a wine fortified with 2.0 mg/L IBMP. An aliquot of 50 mL of wine was loaded onto a conditioned column. Matrix components were rinsed off with 1 mL of a 20% solution of acetonitrile in water while the analyte was eluted with three 1 mL aliquots of acetonitrile. Solvent removal and reconstitution was as before, thus producing a concentration factor of 50. It should be noted that the phase has the capacity to extract at least 500 mL of wine and that larger concentration factors may be realized. The average recovery of IBMP from the wine matrix was 84% (RSD = 8.3%, n = 3).

3.3.4.4. SPE on normal phase silica sorbents

In normal phase (NP) chromatography, polar compounds are adsorbed from a relatively apolar environment onto a polar stationary phase.[2] On the unmodified silica phases, the primary interaction is polar, but the silanol groups are ionizable and can also be utilized as a weak cation exchanger. NP-SPE was evaluated for removal of apolar interferences by retention of the relatively polar 3-alkyl-2-methoxypyrazines. The polarity of water generally precludes the use of silica SPE columns for the extraction of polar compounds from an aqueous environment.

The silica SPE cartridge was conditioned by applying 1 mL of isooctane. Aliquots of 10 mL of fortified wine were then applied to the column and the effluent collected separately for liquid chromatographic analysis. The capacity of the cartridge was determined and analyte breakthrough was observed after 20 mL of wine was loaded onto the column. This relatively low capacity of the silica phase may be ascribed to an unfavorable partitioning ratio of the solute between the polar, aqueous wine matrix and the polar stationary phase. It may therefore be concluded that the phase is not saturated, but rather that partitioning of the solute towards the aqueous sample matrix predominates with the result that the solute elutes without significant retention on the stationary phase.

Matrix removal was evaluated by loading 10 mL of fortified wine onto a single cartridge and applying 1 mL aliquots of wash solvents of increasing polarity. Only water-soluble solvents were evaluated to ensure compatibility with the HPLC method. The effluent was collected separately for HPLC-UV analysis to determine the fractions that contained eluted analyte. The results were as follows (Table 3.6.):

Table 3.6.: Determination of optimal conditions for sample clean-up and pre-concentration utilizing normal phase SPE.

Wash Solution	Peak area (pA.s)
Tetrahydrofuran	11.6
Acetone	5.6
Acetonitrile	3.1
Methanol	-
Water	-

It can be seen that tetrahydrofuran, the least polar, water-soluble solvent investigated, eluted IBMP from the column. Effective removal of wine matrix components from the column is therefore not possible under the described conditions. The efficiency of the silica SPE procedure was considered too low for effective pre-concentration of IBMP from wine and was not further evaluated.

3.3.4.5. SPE on strong and weak cation exchange sorbents

Cation exchange sorbents may be used to extract positively charged (basic) compounds from aqueous samples via ionic interactions between the charged solute and the conjugated base of the stationary phase. Residual silanol groups on the silica support provide secondary polar interactions while the aliphatic or aromatic links may present secondary apolar interactions. Retained cationic compounds may be eluted with a solution of high ionic strength, by neutralizing the charge on the cationic solute, or by eliminating the charge on the stationary phase. Due to secondary apolar interactions, the addition of an organic modifier may enhance elution of the solute.

Strong cation exchange sorbents generally consist of sulfonic acid groups bonded to a silica support via aliphatic or aromatic links. The sulfonic acid group is characterized by a very low pKa (< 1.0) with the result that it remains ionized even at low pH. Weak cation exchange sorbents consist of an aliphatic carboxylic acid bonded to a silica support.[9] The carboxylic acid functional group posses a pKa of approximately 4.8 and is generally used to extract basic compounds capable of carrying a positive charge at pH 6.8 or higher. Elution of the solute from the column may be achieved utilizing the same strategies discussed for the strong cation exchangers.

3.3.4.5.1 Optimization of an SPE procedure on a strong cation exchange sorbent

The Strata SCX cartridge was conditioned by applying 1 mL of methanol followed by 1 mL of water. It is assumed that the amine functional groups of pyrazines are protonated and positively charged in the wine matrix which has a typical pH ranging between 3 and 4.[17] Aliquots of 10 mL wine, fortified to 2.0 mg/L, were applied to various columns followed by a rinsing step with 1 mL water. Analyses

of the effluents revealed complete retention of the solute. A variety of strategies for elution of the solute from the column were then evaluated.

- Elution with concentrated acid (three 1 mL portions of 2 N sulfuric acid). The proposed mechanisms are a mixture of displacement of the solute by a concentrated solution of ions and elimination of the charge on the stationary phase by protonation of the sulfonic acid groups at low pH (the pH of the 2 N sulfuric acid solution is approximately 0.7).
- Elution with concentrated acid with added organic phase (three 1 mL portions of 50% 2 N sulfuric acid in methanol). Possible elution mechanisms involved are ionic strength, neutralization of the charge on the stationary phase as well addition of an organic modifier to compensate for secondary apolar interactions.
- Elution with strong base (three 1 mL portions of 2 N sodium hydroxide). Proposed mechanisms are a mixture of displacement of the solute by a concentrated solution of ions as well as neutralizing the charge on the cationic solute. The pH of the 2 N sodium hydroxide solution was approximately 14.
- Elution with a strong base with added organic phase (three 1 mL portions of a solution of 50% 2 N sodium hydroxide in methanol). Possible elution mechanisms are ionic strength, neutralization of the charge on the solute as well addition of an organic modifier to compensate for secondary apolar interactions.

It was found that the solute is not eluted from the column with any of the proposed solvent systems, indicating that the protonated tertiary di-amine functional group of methoxypyrazines is very strongly retained on this phase. De Boubee *et.al.*[13] successfully rinsed the solute from a small mass of cation exchange resin with 10% NaOH, as opposed to ~8% used in this study in conjunction with 500 mg cartridges. Strong ion exchangers are also known to irreversible retain very strong ions such as quaternary amines.[18]

3.3.4.5.2. Extraction of methoxypyrazines from the distillate of a wine sample utilizing a strong cation exchange sorbent

Several authors reported the successful use of cation exchange resin for the isolation of IBMP from the distillates of wine samples.[4,5,13,15] As this mechanism was anticipated to provide excellent clean-up of extracts for subsequent reversed phase separation, this combination was evaluated. As a relatively large, concentrated NaOH solution is required for elution of the analyte from the SCX resin, back-extraction into dichloromethane is essential. Unlike with with gas chromatography, these extracts then need to be evaporated to remove the dichloromethane followed by reconstitution in mobile phase so that the solutions may be compatible with reversed phase liquid chromatographic separation.

An aliquot of 100 mL of the fortified wine sample (200 ng/L) was distilled utilizing simple distillation and 50 mL of the distillate was collected. An aliquot of 1 mL of sulfuric acid was added and the solution reconstituted to 100 mL with water. The 500 mg SCX column was conditioned by applying 1 mL of methanol followed by 1 mL of water. The total volume of the acidified distillate (100 mL) was then applied to the column. The capacity of the column was determined by analyzing the fraction corresponding to the last 2 mL of distillate that was passed through the column. It was found that no analyte breakthrough occurred during the sample loading step.

The column was rinsed with 1 ml of a solution of 10% methanol in water. Analysis of the rinse revealed that no measurable quantities of the analyte were removed during this step.

Optimal conditions for elution of the analyte from the column were determined by applying 1 mL aliquots of a solution of 5% sodium hydroxide in 50% methanol to

the column. Residues were extracted (from elution solution) into dichloromethane. Aliquots of 0.5 mL concentrated formic acid were then added to the dichloromethane fractions followed by evaporation to less than 0.5 mL. Extracts were then reconstituted in 2.5 mL of water for analysis. From the results it was evident that elution of the solute may be affected with at least six 1 mL aliquots of 5% sodium hydroxide in 50% methanol.

The optimal conditions for the extraction of the analyte from the elution solution (5% sodium hydroxide in 50% methanol) were then determined by performing repeated extractions with 2.5 mL dichloromethane. It was found that three extractions with 2.5 mL dichloromethane ensure complete recovery of the solute from the elution solution.

The efficiency of the optimized distillation/SPE (SCX) procedure was evaluated by determining the average recovery of IBMP from the distillate of fortified wine samples (200 ng/L). Aliquots of 100 mL of wine were distilled and 50 mL of the distillate collected. Aliquots of 1 mL sulfuric acid were then added to each distillate prior to reconstitution to 100 mL with water. These solutions were applied to pre-conditioned SCX cartridges. The collection vessels were rinsed with water and the water passed through the respective columns. After rinsing the cartridges with 1 mL 10% methanol in water, the analyte were eluted with five 2 mL aliquots of 5% sodium hydroxide in 50% methanol. The eluents were subsequently extracted with three 2.5 mL aliquots of dichloromethane. To the dichloromethane fraction were then added 0.5 mL concentrated formic acid upon which the fraction was evaporation to 0.5 mL. Finally the extracts were reconstituted in 1 mL of water for analysis, yielding a concentration factor of 100. Even though the average recovery (n = 3) was 95%, the repeatability of this procedure was very poor, as reflected by an RSD value of 43%. It is anticipated that the poor repeatability of this procedure results from the extraordinary large number of steps that are required to obtain the extract in a solution that is compatible with reversed phase liquid chromatographic separation. When

dissolved in the strong sodium hydroxide solution, the IBMP molecule is also expected to be uncharged and relatively volatile. Moreover, it is expected that the high ionic strength of the sodium hydroxide solution may enhance partitioning of the neutral analyte into the vapor phase, similarly to ionic strength adjustment for SPME sampling. Losses by volatilization of the analyte may thus occur during this step.

3.3.4.5.3. SPE on weak cation exchange sorbents

A weak cation exchange cartridge was conditioned by applying 1 mL of methanol followed by 1 mL of water. As before, it can be assumed that methoxypyrazines are protonated in the wine matrix.[17] An aliquot of 10 mL of fortified wine containing 2.0 mg/L of IBMP, was applied to the cartridge, followed by rinsing with 1 mL water. Analyses of the effluents revealed that the solute passed through the column un-retained under these conditions. Since the pH of wine is below the pKa of the carboxylic acid functional group of the stationary phase, it is presumed that protonation of the stationary phase is responsible for this observation. This precludes the use of this phase for the extraction of the solute from wine without pH adjustment.

The experiment was therefore repeated with a fortified wine of which the pH was adjusted to approximately 9 by the addition of 5% (m/v) sodium hydroxide. Analysis of the effluent revealed that practically no retention is achieved under these conditions. One possible explanation for this result is that the charge on the aromatic amine groups is eliminated under these conditions, with the result that the (uncharged) analyte passes through the cation exchange column un-retained. In conclusion, it was found that the weak cation exchange sorbent was unsuitable for the extraction of IBMP from wine samples.

3.3.5. Evaluation of stir bar sportive extraction (SBSE) for the isolation and pre-concentration of IBMP from wine

SBSE is often used for headspace sampling of volatile analytes in conjunction with thermal desorption and subsequent gas chromatographic analysis.[17] The technique is however equally amenable to liquid sampling as well as liquid desorption, with the result that it can be used with liquid chromatography as well.[8]

The efficiency of SBSE utilizing a polydimethylsiloxane (PDMS) phase was evaluated for sorption of IBMP from wine samples. The technique was evaluated for direct extraction by stirring in wine samples as well as sorption of volatile material from the headspace of an agitated wine sample. PDMS is an apolar phase and is expected to display affinity towards IBMP.

Since extraction in SBSE is based on the partitioning into the PDMS phase, and therefore an equilibrium process, extraction time of 1 hour was allowed for both absorption- and desorption steps. The stir bar was re-activated between experiments by thermal desorption at 220°C for three hours under constant nitrogen flow of 175 mL/min. Recovery of IBMP from the wine samples was determined by analyzing the wine before and after extraction as well as the extract after liquid desorption of the stir bar.

3.3.5.1. SBSE in headspace mode

An aliquot of 5 mL of fortified wine (2.0 mg/L IBMP) and 1.5 g of sodium chloride were transferred to a 25 mL vial containing a small Teflon-coated stir-bar. The PDMS-coated stir-bar was placed in the headspace of the sample utilizing a glass adapter, upon which the vial was sealed. The sample solution was stirred for 1 hour during the extraction step. The stir-bar was subsequently removed and

IBMP was desorbed by stirring for 1 hour in 1 mL of solvent. Two desorption solvents were evaluated, acetonitrile and dichloromethane, the recovery of IBMP are summarized in Table 3.7.

Table 3.7.: Recovery of IBMP from the headspace of an agitated (and saturated with sodium chloride) wine sample.

Extraction	IBMP residue in wine after extraction (%)	Recovery of IBMP (%)
1	12.4	2.5 (acetonitrile)
2	18.0	2.4 (acetonitrile)
3	29.1	6.1 (dichloromethane)

From these results it can be concluded that even though partitioning of IBMP into the stir bar seems to be favored from the saturated (sodium chloride) wine, subsequent recovery of the analyte by liquid desorption was very poor. Two possible explanations for the poor recovery are that the IBMP are forced out of solution by ionic strength adjustment but that sorption onto the stir bar is unfavorable due to competition with wine ethanol as well as possible unfavorable partitioning between the PDMS phase and the desorption liquids.

3.3.5.2. SBSE in submersion mode

An aliquot of 5 mL wine, fortified with 2.0 mg/L IBMP as well the PDMS-coated stir bar was sealed inside a 25 mL vial. The sample was stirred, at a relatively fast rate, for 1 hour upon which the stir bar was removed, rinsed with water, dried on tissue paper. The analyte was desorbed by stirring for 1 hour in 1 mL acetonitrile or dichloromethane. The results of the recovery study are presented in Table 3.8.

Table 3.8.: Recovery of IBMP from a wine sample (direct stirring).

Extraction	IBMP residue in wine after extraction (%)	Recovery of IBMP (%)
1	84.1	10.0 (acetonitrile)
2	No result.	25.0 (acetonitrile)
3	86.1	7.6 (dichloromethane)

These results indicate that partitioning of the relatively polar IBMP into the PDMS phase is not favored. Subsequent liquid extraction provided relatively good removal of the extracted analyte, in contrast to the result obtained for headspace sampling. It can be concluded that SBSE (in both headspace and immersion modes) in combination with liquid desorption is not an effective sample pretreatment step for the analysis of methoxypyrazines in wine samples by HPLC.

A possible explanation for the poor performance of SBSE may be that the ethanol content of the wine interfered with efficient sorption of IBMP into the phase. Successful application of the SPME technique, utilizing PDMS-DVB fibers, was reported where the wine was acidified and distilled to remove the ethanol followed by neutralization and ionic strength adjustment, prior to extraction.[6]

3.4. Conclusions

Various sample clean-up and pre-concentration strategies were evaluated including distillation, solvent extraction and solid phase extraction.

It was demonstrated that dichloromethane solvent extraction is capable of quantitative extractive pre-concentration of mg/L levels of IBMP from Sauvignon blanc wine. The average recovery was better 90% with RSD of 11%, while a concentration factor of 200 was attained utilizing direct solvent extraction with

dichloromethane. A total of 25 mL of dichloromethane is used in the process with total equilibration time of 60 minutes. Total time per sample is 2 hours.

Distillation may be used in tandem with the described solvent extraction procedure to improve the efficiency of the sample preparation procedure. Distillation imparts an extra measure of selectivity to the process thus reducing the matrix of the extract. The distillates also require less solvent for extraction with reduced equilibration time. As the evaporation of solvent from the extract is very time consuming, reduction of the amount of solvent required will make the process more time-efficient. The efficiency of the sample preparation procedure is also improved as larger effective volumes of sample may be extracted resulting in increased concentration factors. When used in tandem, the average recovery of the combined processes was 110% with RSD of 12% while yielding a concentration factor of 500. A total of 20 mL of dichloromethane is used in the process with total equilibration time of 30 minutes. Total time per sample is 90 minutes.

When solvent extraction is performed on the distillate, the mixture can be stirred at a fast rate thereby speeding up the process. The dichloromethane extracts must not be evaporated to complete dryness as losses of IBMP then occur while the addition 0.5 mL concentrated formic acid prior to evaporation ensures protonation of the analyte thereby rendering it less volatile (ionic state less volatile than neutral molecule).

The efficiency of various SPE phases was evaluated for direct extraction of IBMP from the wine matrix. Table 3.9. summarizes the results obtained with various phases.

Table 3.9.: Efficiencies of various SPE phases for the extraction and pre-concentration of IBMP.

Sorbent chemistry	Capacity (µg/g sorbent)	Average recovery (%)	RSD (%)
C18, endcapped	147	88	6.1
Oasis HLB	630	79	5.1
Polystyrene-divinylbenzene	> 479	84	8.3
Unmodified silica	-	-	
Strong cation exchange	-	-	
Weak cation exchange	-	-	

Those phases designed to retain compounds of low to medium polarity via a mixture of polar and apolar interactions were found to be more efficient. The procedure used with Oasis HLB cartridges was optimized to enhance the retention of basic compounds and proved to be particularly efficient. Non-endcapped C18 phases may also possibly produce better results than endcapped phases due to secondary polar interactions provided.

Two SPE phases were also evaluated for the extraction of IBMP from the distillate of wine samples. The results are presented in Table 3.10.

Table 3.10.: Efficiencies of various SPE phases for the extraction of IBMP from the distillates of wine samples.

Sorbent chemistry	Concentration factor achieved.	Average recovery (%)	RSD (%)
Oasis HLB	500	82	4.3
Strong cation exchange	100	95	43

Oasis HLB cartridges provided very efficient clean-up and pre-concentration of the distillate. The SCX procedure did not produce satisfactory results and required a large number of steps to ensure compatibility of the extract with the analytical liquid chromatographic column.

The PSDVB phase provided the capacity to extract the required volume of wine (500 mL) to affect a concentration factor of 500, but the practicality of loading this volume on standard SPE cartridges generally rendered this technique unsuitable for use in this study. Oasis HLB produced the same concentration factor when utilized to extract the reconstituted (2% aqueous ammonia) dichloromethane extract of a (distilled) wine sample and provided very efficient sample clean-up. The procedure was however very elaborate and did not remove critical interferons as was evident upon C18 separation of the extracts and APCI-LC-MS analysis.

The efficiency of the polydimethylsiloxane coated stir-bar for the absorption of IBMP from wine samples was very low. Sala *et.al.*[6] reported the successful use of polydimethylsiloxane-divinylbenzene coated fibers in the headspace solid-phase microextraction of IBMP from wine with excellent recovery. The procedure described however involves distillation of an acidified (pH 0.5) wine sample to remove ethanol and other volatile interfering matrix components with subsequent neutralization, in conjunction with high ionic strength, for headspace extraction of various 3-alkyl-2-methoxypyrazines. It may therefore be assumed that that ethanol and other wine matrix components, that were not removed prior to extraction in this study, prohibited efficient recovery of IBMP from unmodified wine samples by saturation of the phase.

It can be concluded that fractional distillation (60 cm column) followed by dichloromethane solvent extraction produced very efficient pre-concentration (a factor of 500) of wine samples. The recovery obtained was 110% with RSD of 12%. Additional sample clean-up was achieved by further extracting the sample solution with Oasis HLB SPE columns, but this degraded the precision (82% recovery, RSD = 4.3%) and time-efficiency of the procedure. The procedure utilizing distillation in combination with dichloromethane solvent extraction as described in section 3.3.3.2. was considered as most suited for use in the investigation.

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CHAPTER 4

Development of an optimized liquid chromatography - mass spectrometric method for trace level quantitation of selected 3-alkyl-2-methoxypyrazines

4.1. Introduction and objectives

The objective of this Chapter is to describe the development of an optimized liquid chromatography mass spectrometric (LC-MS) method for sensitive and selective measurement of selected 3-alkyl-2-methoxypyrazines in wine namely 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP), 3-ethyl-2-methoxypyrazine (EMP) and 3-methyl-2-methoxypyrazine (MMP). As the expected levels of the analytes in wine are very low, a sample preparation step affecting pre-concentration of a factor of 500 (described in Chapter 3) was used. Extracts were separated utilizing reversed phase liquid chromatography followed by atmospheric pressure ionization for mass spectrometric analysis. To facilitate accurate trace level quantitation of the components of interest in the presence of concentrated matrix elements, experiments were aimed at determining the optimal experimental parameters pertaining to the separation, ionization, fragmentation and detection of the substances under investigation.

4.2. Experimental

4.2.1. Materials

Only high purity solvents and chemicals were used throughout the experiment. Acetonitrile, dichloromethane and tannic acid were from Merck (Darmstadt, Germany), IBMP, IPMP, SBMP, EMP, MMP, methanol and ethanol from Aldrich (Sigma-Aldrich, South Africa) hexane from Burdick & Jackson (Honeywell International, U.S.A.) and formic acid from Associated Chemical Enterprises (ACE, South Africa).

Standards were prepared by weighing appropriate amounts of reference material on an analytical balance followed by dilution utilizing A-grade volumetric glassware. Intermediate standards were prepared in ethanol while working standards were prepared in 10% ethanol. For the exploratory work, where the efficiency of octadecyl and polystyrene-divinylbenzene columns were compared utilizing LC-UV, working standards were prepared in 50% acetonitrile to correspond approximately to the gradient starting concentration. Wine samples for the recovery studies were fortified by adding appropriate amounts of standards followed by distillation, solvent extraction and pre-concentration as described in Chapter 3.

4.2.2. Instrumentation

The LC-MS system utilized was a Waters Alliance 2695 liquid chromatograph (Waters Corporation, Milford, U.S.A.), equipped with a six-port switching valve and Waters Micromass Quattro Premier XE tandem quadrupole mass spectrometric detector (Manchester, U.K.). Nitrogen gas for the atmospheric-pressure interface was supplied by a generator while bottled argon (5.0) was used as collision gas. Post-column addition of reagents was done with a

Shimadzu LC-6A liquid chromatography pump and Shimadzu SCL-6B system controller (Shimadzu Corporation, Kyoto, Japan), synchronized with the LC-MS system. Analytes were ionized utilizing either of two atmospheric pressure ionization techniques namely electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Mass spectral information was acquired by direct infusion of standards of 3 to 8 mg/L of the components of interest via an integrated syringe pump.

For the preliminary evaluation of the efficiency of octadecyl and polystyrene-divinylbenzene columns, an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) fitted with quaternary pump, autosampler, column oven and UV-visible diode array detector was used. The mobile phase consisted of various acetonitrile/water gradients. The columns were a Luna C18 (150 x 4.6 mm) with 3 µm particles and a PolymerX polystyrene-divinylbenzene column (150 x 4.1 mm) with 3 µm particles (both Phenomenex, Torrance, U.S.A.). The column-temperature was 30°C and variable injection volumes were used. The chromatographic signal at 292 nm was acquired while spectra between 190 and 400 nm were recorded. All columns evaluated during the course of the study are summarized in Table 4.1.

A Waters API Q-TOF Ultima LC-MS system was used to perform accurate mass determinations of the molecular- and fragment ions of the methoxypyrazines. Ionization was performed using positive ESI with a capillary voltage of 3.5 kV. The cone voltage was 35 V while the source and desolvation temperatures were 100 and 250 °C respectively. Desolvation gas flow was 200 L/h and cone gas flow 50 L/h. The collision energy for MS/MS mode of operation was 20 eV.

Table 4.1.: Description of columns used in the investigation.

Phase	Manufacturer / brand name	Dimensions
C18	Phenomenex Luna	150 x 4,6 mm, 3 µm
Polystyrene-divinylbenzene	Phenomenex PolymerX	150 x 4,1 mm, 3 µm
Phenyl hexyl	Phenomenex Luna	250 x 4,6 mm, 5 µm
C8	Agilent Xorbax Eclipse XDB C8	150 x 4.6 mm, 5 µm
C5	Phenomenex Luna	150 x 4,6 mm, 5 µm
CN	Phenomenex Luna	250 x 4,6 mm, 5 µm 50 x 4.6 mm, 5 µm

4.3. Results and discussion

4.3.1. Initial selection of chromatographic separation mode

The 3-alkyl-2-methoxypyrazines under investigation are relatively apolar molecules and are expected to be retained strongly on reversed phase columns. Strong solute retention may facilitate elution with a mobile phase that contains a relatively high proportion of organic modifier as well as potential separation of the solute from matrix interferences via a gradient. Efficient removal of the elution solvent during the ionization process is critical for optimal introduction of the ions into the mass spectrometer and hence method sensitivity. Solvent evaporation and ionization are also more efficient from a mobile phase that contains a higher proportion of an appropriate volatile organic modifier. Moreover, co-eluting matrix elements may interfere with the efficient ionization and mass spectrometric determination of target compounds and separation of interferences may therefore be critical for method performance. For this reason the initial part of this investigation focused on the choice of optimal chromatographic conditions, taking into account these considerations.

4.3.1.1. Evaluation of C18 and polystyrene-divinylbenzene phases for the reversed phase separation of various 3-alkyl-2-methoxypyrazines

The efficiency of octadecyl and polystyrene-divinylbenzene phases was evaluated for the reversed phase separation of five 3-alkyl-2-methoxypyrazines. The objective was to identify the phase that offers the best resolution as well as strongest retention of the analytes, with consequent higher proportion of organic modifier in the mobile phase at the time of elution.

A standard was analyzed by the liquid chromatographic method with UV detection described in the experimental section. The gradient used for elution of the analytes from the C18 column was as follows: Initially 50% acetonitrile for 30 seconds, 50 to 95% acetonitrile in 7 minutes, 95% acetonitrile for 4 minutes. The flow-rate in the separation was deliberately restricted to 0.5 mL/minute as it was envisaged that this separation would eventually be used in conjunction with electrospray ionization and therefore necessitate lower flow rates.

As expected, the degree of retention on the apolar phases increased with the number of carbon atoms and their arrangement, in the bonded alkyl chain. The order of elution was MMP, EMP, IPMP, IBMP and SBMP (Figure 4.1.).

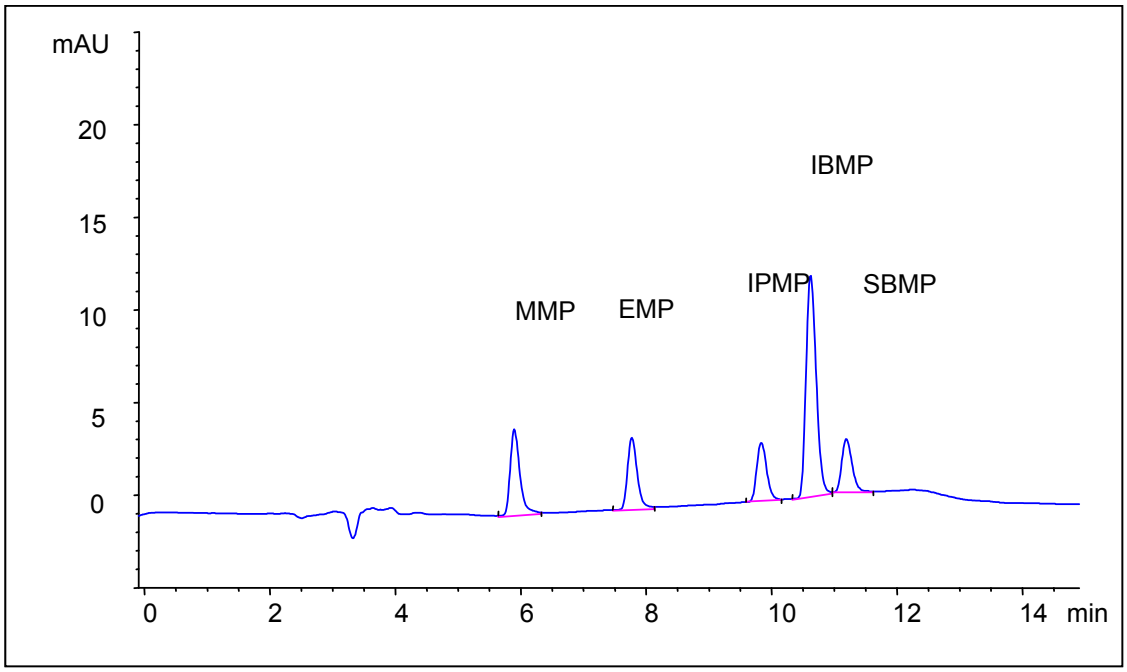


Figure 4.1.: Standard of MMP (27 ng), EMP (26 ng), IPMP (25 ng), IBMP (96 ng), SBMP (24 ng), in 50% acetonitrile, C18 phase, 150 x 4.6 mm x 3 µm. Signal recorded at 292 nm.

The gradient used for elution of the analytes from the polystyrene-divinylbenzene phase started at 45% acetonitrile for 1 minute, increased to 75% acetonitrile in 7 minutes, followed by 75% acetonitrile for 2 minutes. The flow-rate was restricted to 0.5 mL/minute for the reasons discussed earlier (Figure 4.2.).

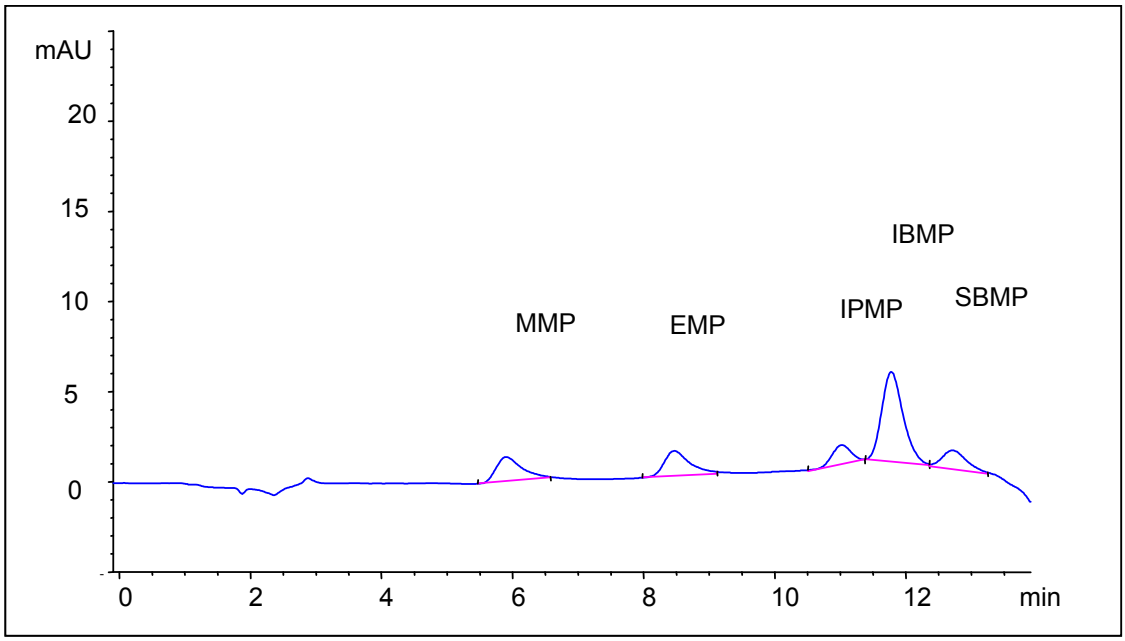


Figure 4.2.: Standard of MMP (27 ng), EMP (26 ng), IPMP (25 ng), IBMP (96 ng), SBMP (24 ng), in 50% acetonitrile, polystyrene-divinylbenzene phase, 150 x 4.6 mm x 3 µm. Signal recorded at 292 nm.

The peaks are clearly better resolved on the C18 phase and the corresponding fraction of organic solvent in the mobile phase is also greater than is the case on the polystyrene-divinylbenzene phase. Any attempt to increase the fraction of organic solvent in the mobile phase in the separation on the latter phase resulted in a severe loss of resolution. The C18 phase was therefore provisionally selected for performing the separations in the investigation. It should be noted that the separations were performed at less than optimal mobile phase flow-rates in both instances as the purpose here was to compare the efficiency of the two phases. As it was envisaged that the separation method would eventually be used in conjunction with electrospray ionization, the evaluation was carried out at relatively low (less than optimal) flow-rates.

4.3.2. Determination of the optimal flow rate in the chromatographic separation utilizing 4.6 mm diameter columns

It is imperative to optimize flow-rates in chromatographic separations in order maximize the plate count and to achieve optimal signal-to-noise ratios for peaks of interest. The optimal flow rate on the C18 column (150 x 4.6 mm) was determined by performing a series of injections of a standard of IBMP at different flow rates. The mobile phase in the isocratic separation consisted of 88% aqueous acetonitrile with 1% formic acid. Mass spectrometric detection with electrospray ionization was utilized in these experiments. The number of theoretical plates (N) for each separation was determined as follows:[1]

$$N = 5.54 (t_R / W_{1/2})^2 \qquad (8)$$

Where t_R is retention time and $W_{1/2}$ the peak width at half-height.[1] It is evident from the results summarized in Figure 4.3. that the optimal flow rate on the 4.6 mm diameter column is approximately 1 mL/minute, correlating with the value expected for small organic molecules.

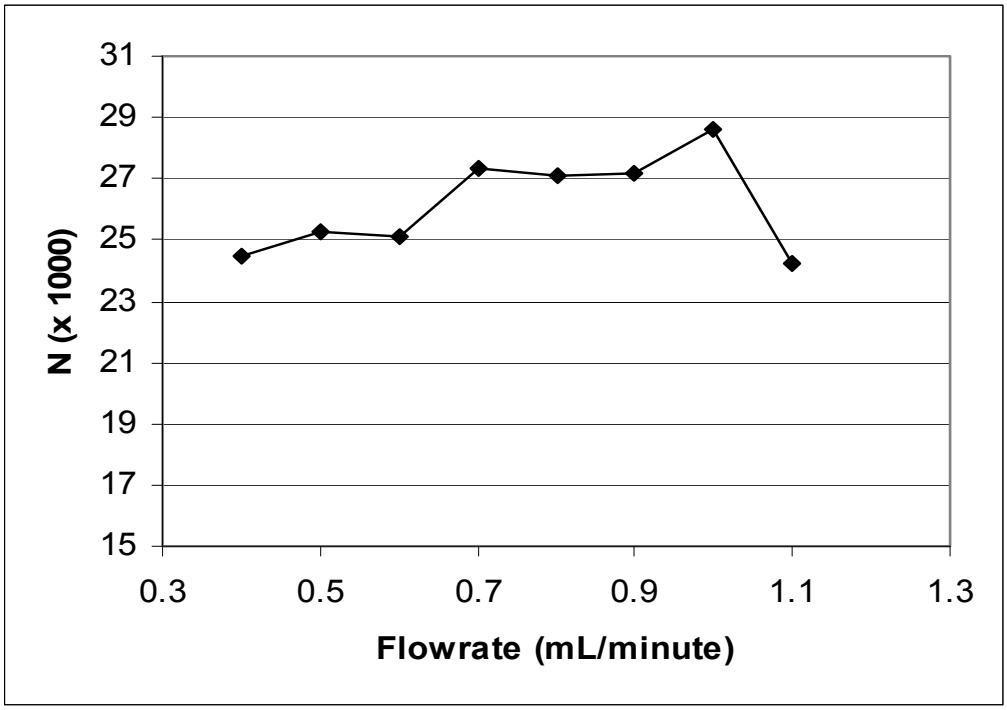


Figure 4.3.: Column efficiency at various flow rates.

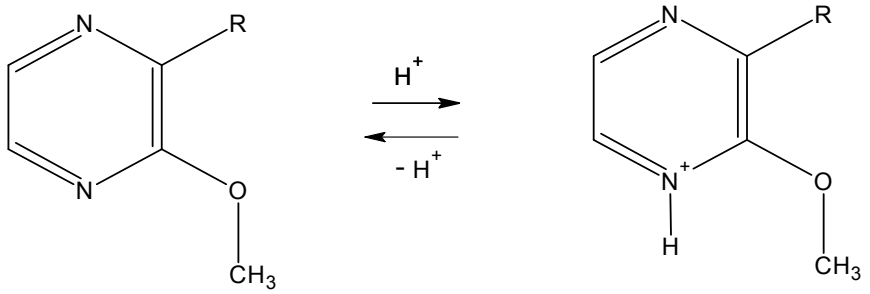
4.3.3. Determination of optimal ionization parameters for the LC-MS analysis of methoxypyrazines

Target compounds, upon elution from the column, need to be ionized in order to draw them into the mass spectrometer for analysis. The interface between the liquid chromatograph and the mass spectrometric detector must be capable of removing most of the elution solvent while efficiently conferring a charge to the target species. The charged species should then selectively be drawn into the mass spectrometer. As the objective of the study is to measure trace level components in a complex matrix, it is imperative to optimize the selective ionization and introduction of these components in order to confer the highest possible level of sensitivity to the method. Table 4.2. presents some relevant chemical data of the substances under investigation:

Table 4.2.: Chemical data of the 3-alkyl-2-methoxypyrazines.[2]

Substance	Molecular formula	Molecular weight (Da)
MMP	$C_6H_8N_2O$	124.14
EMP	$C_7H_{10}N_2O$	138.17
IPMP	$C_8H_{12}N_2O$	152.20
IBMP	$C_9H_{14}N_2O$	166.22
SBMP	$C_9H_{14}N_2O$	166.22

Methoxypyrazines are aromatic bases with pairs of nonbonding electrons available to abstract a proton and form positively charged species. As the protonated, cationic species are still aromatic, it is expected that it will react readily to produce stable cations. The protonation may proceed as follows (Figure 4.4.) with the positive charge residing on the no.1 nitrogen, activated by the methoxy group.[3]



Where R = CH_3 or CH_2CH_3 or $CH(CH_3)_2$ or $CH_2CH(CH_3)_2$ or $CH(CH_3)CH_2CH_3$

Figure 4.4.: Protonation of the 3-alkyl-2-methoxypyrazines.

Two atmospheric pressure ionization techniques capable of positive mode ionization were available with the instrument used in this study, namely electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). As the methoxypyrazines will be measured in multiple reaction monitoring (MRM) mode, it is imperative that the introduction of precursor molecular ions is optimized in order to measure resulting daughter ions at optimal sensitivity.

The efficiency of ESI and APCI was evaluated and compared directly by infusing a 6 mg/L standard of 3-isobutyl-2-methoxypyrazine at 3 µL/minute in conjunction with a flow of 1 mL/minute from the liquid chromatograph using a mobile phase of 1% formic acid in 88% aqueous acetonitrile. The MS response for IBMP was optimized for ESI and APCI simultaneously by observing the intensity of the molecular ion signal while experimental parameters were adjusted to produce the best sensitivity. The signal obtained for ESI was approximately an order of magnitude more intense than that obtained for APCI, although it must be noted that the response thus obtained for APCI using acetonitrile as modifier may not be optimal. Positive mode electrospray ionization (ESI) was therefore initially selected for detection of the methoxypyrazines.

4.3.4. Optimization of positive mode electrospray ionization parameters

ESI is a soft ionization technique suitable for compounds that are readily ionizable and produces little fragmentation. An electrospray is produced upon application of an electric field to a slow flow of conducting liquid.[4] As ESI is concentration independent, this ionization technique should ideally be used with low flow-rates.[5]

4.3.4.1. Effect of the mobile phase organic modifier

The mobile phase in reversed phase separations essentially consists of mixtures of water and an organic modifier. Acetonitrile and methanol are frequently used organic modifiers in reversed phase separations employing mass spectrometric detection.[5,6] Table 4.3. lists some relevant physical properties of these solvents.

Table 4.3.: Physical properties of the organic modifiers studied during the optimization of positive mode electrospray ionization parameters[7,8]

Solvent	Molecular weight (Da)	Boiling point (°C)	Polarity index	Viscosity (m Pa s)
Acetonitrile	41.05	81.7	5.8	0.369
Methanol	32.04	64.6	5.1	0.544
Water	18.02	100.0	9.0	0.890

Electrospray ionization efficiency was evaluated in elution systems of aqueous acetonitrile and methanol. Sample introduction was by direct infusion of a standard (3 mg/L) at 5 µL/minute via a syringe pump in conjunction with a flow of 0.3 mL/minute from the liquid chromatograph at the various mobile phase compositions. The MS response (scan mode) was optimized by observing the intensity of the molecular ion while experimental parameters were adjusted to produce the best sensitivity. Mass spectra obtained for 3-methyl-2-methoxypyrazine in 95% aqueous acetonitrile and methanol are compared in Figures 4.5. and 4.6.

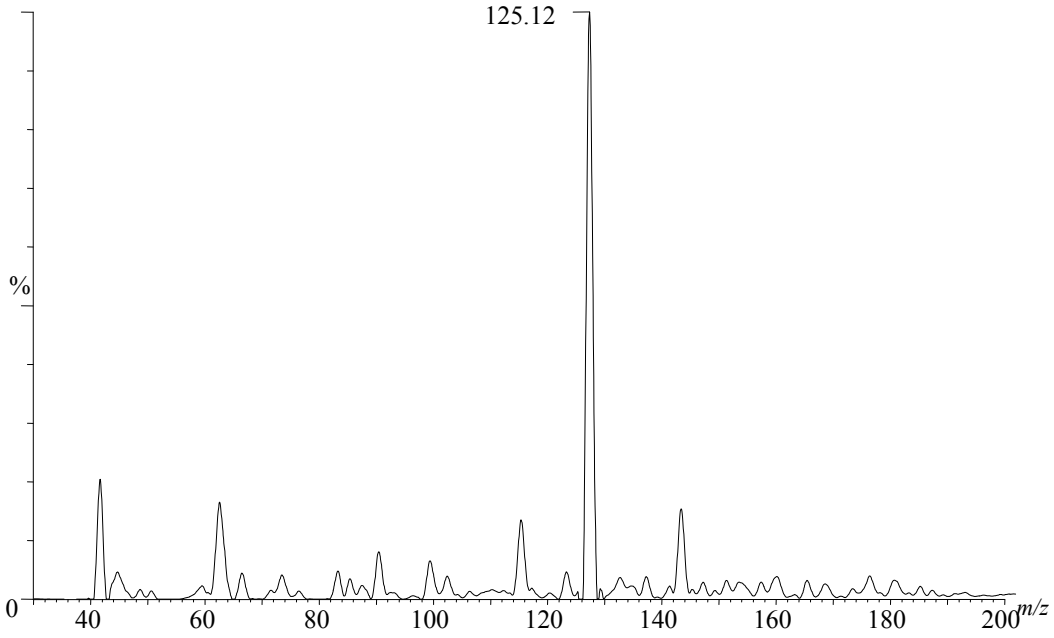


Figure 4.5.: ESI (positive ion mode) mass spectrum for MMP, 95% acetonitrile.

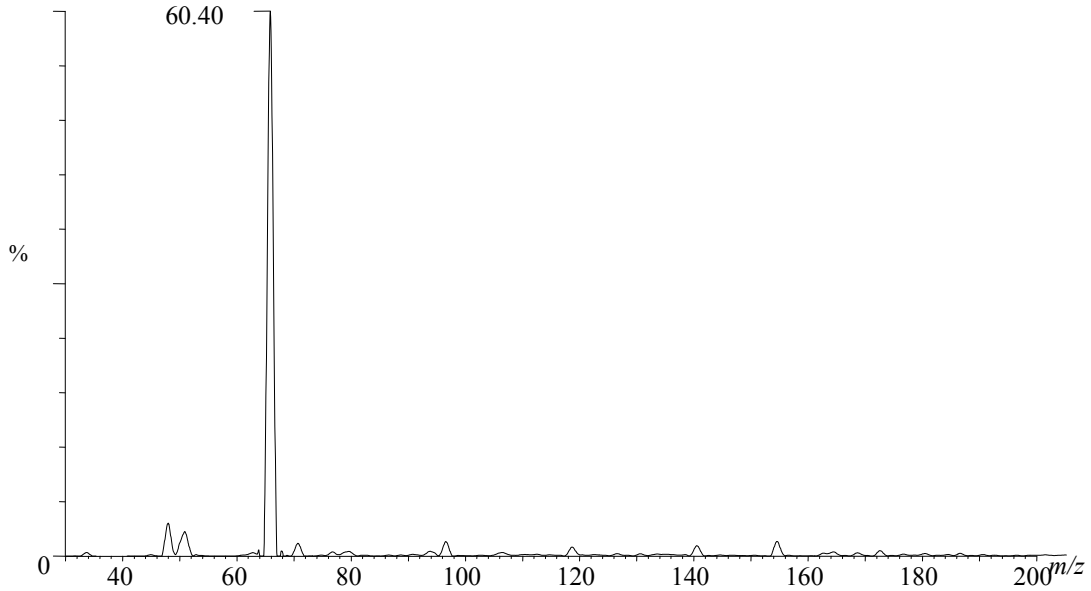


Figure 4.6.: ESI (positive ion mode) mass spectrum for MMP, 95% methanol.

It is evident that electrospray ionization is more efficient utilizing acetonitrile as organic modifier under the MS conditions specified. An intense molecular ion (M + 1) peak (at mass 125) is observed in the mass spectrum obtained using acetonitrile, while the corresponding peak is absent in the spectrum obtained using methanol. Excessive fragmentation or the formation of a doubly charged species may account for the intense peak at mass 60 observed in the spectrum utilizing aqueous methanol.

The wide disparity in electrospray ionization efficiency that was observed between acetonitrile and methanol suggest that the solvents in the elution system play a major role in promoting ionization of methoxypyrazines. Ionization efficiency was therefore evaluated utilizing different solvents which may potentially be used in the reversed phase separation of these compounds. Standards of 8 mg/L MMP were prepared in various solvents, which were then infused directly via the syringe pump at 50 µL/minute, without additional flow from the HPLC. Table 4.4. presents a list of the solvents that were evaluated together with the relative intensities of the resulting molecular ion peak (M + 1).

Table 4.4.: Ionization efficiency of MMP in various solvents.

Solvent	Relative intensity of molecular ion peak
Acetonitrile	116
Ethyl acetate	53
Acetone	20
Tetrahydrofuran	5
Methanol	3
Di-ethyl ether	3
Water	2
0.5% Formic acid in water	12
0.5% Acetic acid in water	7
20 mM Ammonium formate in water	12
20 mM Ammonium acetate in water	10

Of the solvents evaluated, ionization of MMP is most efficient in acetonitrile. Further optimization experiments therefore focussed on acetonitrile as the organic mobile phase modifier utilized in conjunction with electrospray ionization.

4.3.4.2. The effect of the aqueous content of the mobile phase on ionization efficiency

As demonstrated earlier, the 3-alkyl-2-methoxypyrazines under investigation may be separated efficiently utilizing reversed phase chromatography. The selected separation involves gradient elution where the aqueous fraction of the mobile phase is varied from 50% to 5% during the chromatographic run. The effect of the varying aqueous content of the mobile phase on the ionization efficiency utilizing ESI was evaluated by acquiring MS scan data while increasing the aqueous content of the mobile phase. The response measured for mass 125 (3-methyl-2-methoxypyrazine) at various mobile phase compositions is shown in Figure 4.7.

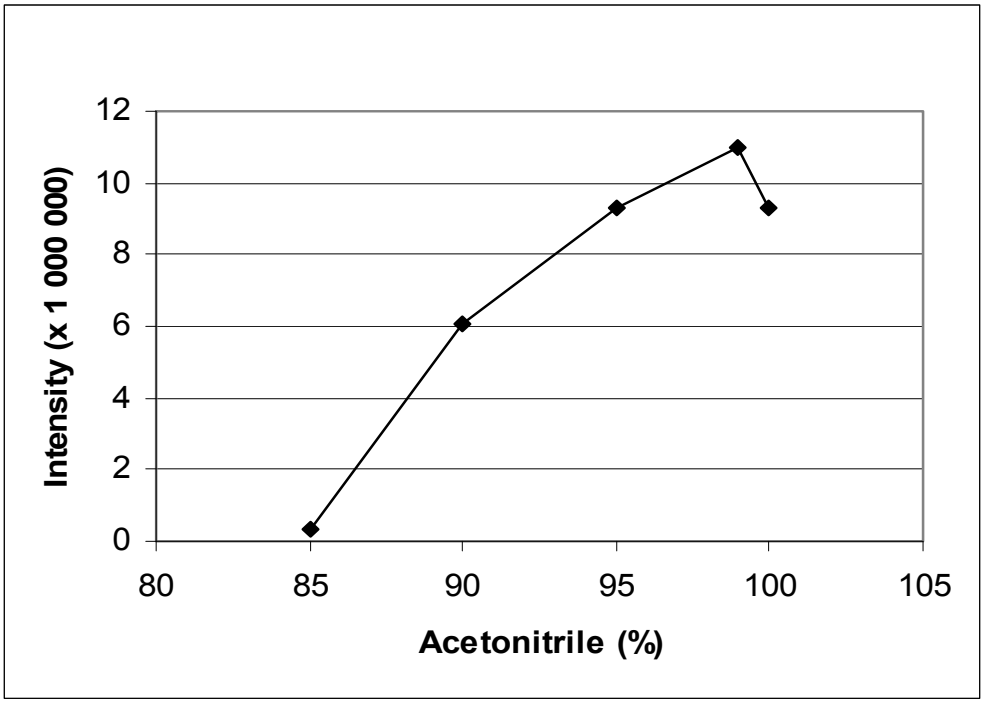


Figure 4.7.: Intensity of M + 1 peak of MMP obtained with (positive mode) ESI at various mobile phase compositions of acetonitrile and water (100%, 99%, 95%, 90% and 85% acetonitrile respectively).

The ionization efficiency decreased when increasing the aqueous content of the mobile phase above 5%. It was observed that in 85% aqueous acetonitrile, the molecular ion peak (M + 1) is completely absent from the relevant spectrum.

4.3.4.3. The use of a volatile buffer to promote ionization

As protonation of the compound of interest is a premise for efficient positive mode electrospray ionization, the use of formic acid to promote ionization was investigated.

For this investigation, sample introduction was by direct infusion of a standard of 3 mg/L at 5 µL/minute via a syringe pump, in conjunction with a flow of 1

mL/minute from the liquid chromatograph. The quaternary solvent delivery system was utilized to produce mobile phase compositions of varying acetonitrile, water and formic acid content. The relative intensity of the molecular ion at each mobile phase composition was obtained from the relevant mass spectra. Initial investigations were performed using 3-methyl-2-methoxypyrazine, as this compound is relatively weakly retained in the reversed phase separation and hence elutes in mobile phase with a relatively high aqueous content. Figure 4.8. presents the relative intensities of the molecular ion (M + 1, m/z = 125) at each mobile phase composition for 3 mg/L MMP.

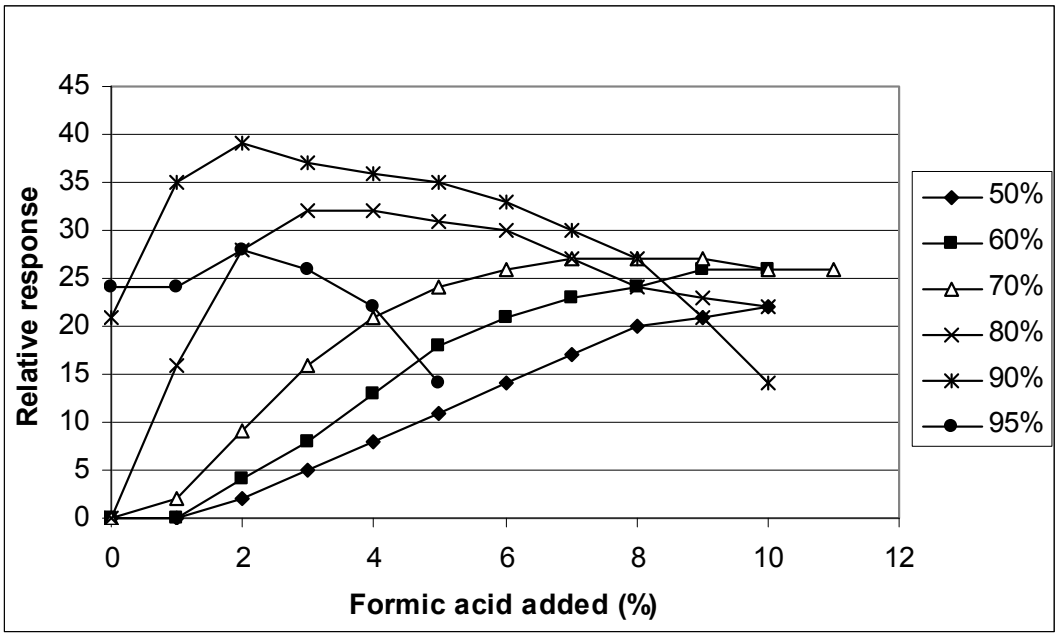


Figure 4.8.: ESI Ionization efficiency of MMP in mobile phase compositions ranging from 50% to 95% acetonitrile and 0% to 11% formic acid.

The addition of formic acid to the mobile phase affected an increase in the ionization efficiency across the entire range of mobile phase compositions evaluated. The optimal amount of formic acid decreases as the fraction of acetonitrile in the mobile phase increases, while ionization efficiency increases with the acetonitrile content of the mobile phase (as observed previously). The

most intense signal was observed using a mobile phase consisting of 90% acetonitrile with 2% formic acid.

As optimal ionization efficiency was obtained between 80 and 90% acetonitrile with between 2 and 4% formic acid, additional experiments were performed in this range of mobile phase compositions. 3-Isobutyl-2-methoxypyrazine is the most significant congener in Sauvignon blanc wine and is expected to elute with the other major 3-alkyl-2-methoxypyrazines, IPMP and SBMP, at higher organic modifier content of the mobile phase. Subsequent experiments were therefore aimed at optimizing ionization of this compound. Figure 4.9. presents the relative intensities of the molecular ion (M + 1, m/z = 167) at intermediate mobile phase compositions for 6 mg/L IBMP infused at 5 µL/minute.

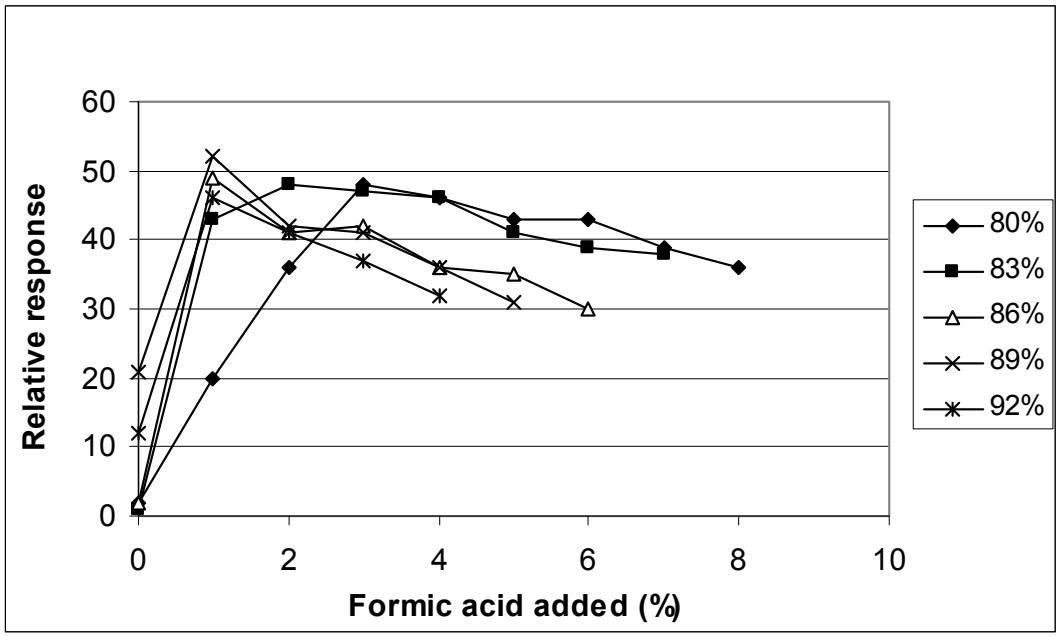


Figure 4.9.: ESI Ionization efficiency of IBMP in mobile phase compositions ranging from 80% to 92% acetonitrile and 0% to 8% formic acid.

It was found that optimal ionization of IBMP was obtained between 86% and 92% acetonitrile with the addition of 1% formic acid. Further experiments at mobile phase compositions of between 85 and 90% acetonitrile with 1% added formic

acid were performed. Figure 4.10. presents the relative intensity of the molecular ion (M + 1) at intermediate mobile phase composition for 6 mg/L 3-isobutyl-2-methoxypyrazine.

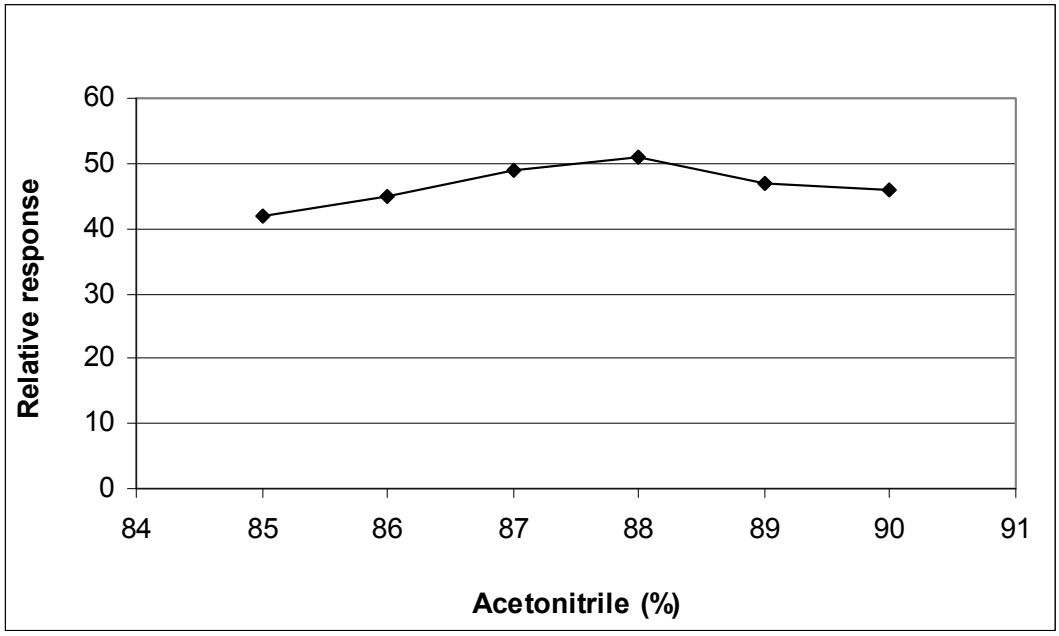


Figure 4.10.: Ionization efficiency of IBMP in mobile phase compositions ranging from 85% to 90% acetonitrile with 1% added formic acid, measured as the intensity of the molecular ion.

The optimal amount of acetonitrile with a constant addition of 1% formic acid was therefore determined to be 88%. The optimal fraction of formic acid at this mobile phase composition was then determined by performing additional experiments while varying the amount of formic acid (Figure 4.11.).

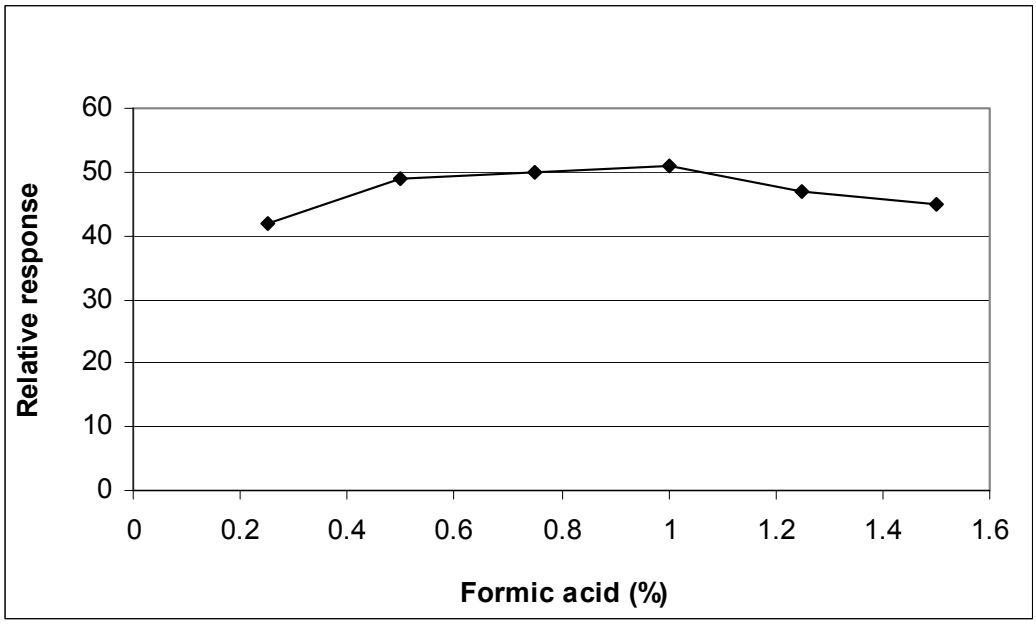


Figure 4.11.: Ionization efficiency of IBMP in mobile phase compositions ranging from 0.25% to 1.50% formic acid in 88% aqueous acetonitrile.

Electrospray ionization was therefore found to be optimal in a mobile phase consisting of 1% formic acid in 88% acetonitrile. The variation in ionization efficiency observed may be rationalized by considering that at the relatively high flow-rates used (1 mL/minute), evaporation was more efficient in a mobile phase that contains a relatively high proportion of volatile organic modifier. The increase in efficiency with addition of formic acid that was observed may possibly be ascribed to enhanced conductivity of the mobile phase and more efficient protonation of the analyte at lower mobile phase pH.

4.3.4.4. The effect of mobile phase flow-rate on ionization efficiency

The effect of mobile phase flow-rate on the electrospray ionization efficiency was evaluated by infusing a standard via the syringe pump at 5 µL/minute while varying the flow from the liquid chromatograph between 0.1 and 1.0 mL/minute. A 6 mg/L standard of IBMP was infused together with a mobile phase

composition of 88% aqueous acetonitrile and 1% formic acid, while scanning for the daughter ion at mass 124. It was found that the intensity of the secondary ion increased with flow-rates from 0.1 mL/minute and reached a maximum at around 0.7 mL/minute. The intensity of the signal remained largely unchanged between 0.7 mL/minute and 1.0 mL/minute, indicating that flow-rates of up to 1.0 mL/minute may be used without significant loss in sensitivity (Figure 4.12.).

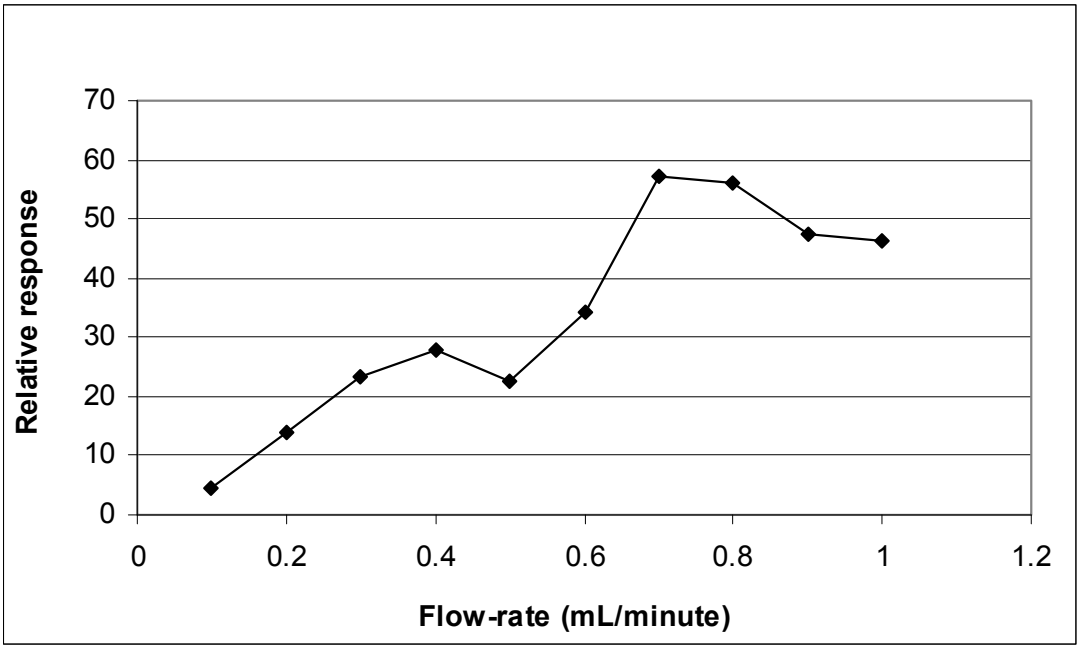


Figure 4.12.: Ionization efficiency at various flow-rates of a mobile phase consisting of 88% acetonitrile and 1% added formic acid.

4.3.4.5. Optimisation of instrument tuning parameters for MS/MS detection

Optimization experiments were performed using approximately optimal tune parameters which were unchanged throughout the method development phase. The mass spectrometer parameters were finally adjusted, with all other parameters at optimal values, to achieve optimal electrospray ionization, introduction, fragmentation and detection. Optimization was performed by

infusing a standard (6 mg/L) of IBMP at 3 µL/minute via a syringe pump, in conjunction with a flow of 1.0 mL/minute from the liquid chromatograph at the mobile phase composition of 88% acetonitrile with 1% formic acid. Various parameters were optimized by observing the intensity of the relevant ions while adjusting each parameter to produce the best sensitivity. Tables 4.5. and 4.6. presents the optimized instrument tuning parameters determined in this manner.[6]

Table 4.5.: Optimized ESI tuning parameters for MS/MS operation determined for mobile phase of 88% acetonitrile with 1% added formic acid.

Parameter	Value
Source: Positive mode electrospray ionization	
Capillary (kV)	2.2
Cone (V)	34
Extractor (V)	6.00
RF Lens (V)	0.1
Source Temperature (°C)	120
Desolvation Temperature (°C)	350
Cone Gas Flow (L/Hr)	50
Desolvation Gas Flow (L/Hr)	650
Analyzer	
LM 1 Resolution	10.0
HM 1 Resolution	10.0
Ion Energy 1	0.3
Entrance	-5
Collision (eV)	18
Exit	0
LM 2 Resolution	14.0
HM 2 Resolution	14.0
Ion Energy 2	1.0
Multiplier (V)	700
Collision Gas Flow (L/Hr)	0.40

The parameters for the MRM transitions were optimized by infusing a standard of 3 mg/L of the five components in conjunction with a mobile phase flow of 1 mL/minute from the HPLC. The mobile phase was 88% aqueous acetonitrile with 1% added formic acid. Experimental parameters were adjusted to produce optimal response for the components of interest. The inter channel delay time was 0.020 seconds while the inter scan time was 0.100 seconds.

Table 4.6.: Optimized parameters for MRM transitions.

Compound	Parent Ion (Da)	Daughter Ion (Da)	Transition	Cone (V)	Collision (eV)
MMP	125.0	56.2	2 °	34	20
	125.0	97.0	1 °	34	14
EMP	139.0	83.0	2 °	34	16
	139.0	124.0	1 °	34	18
IPMP	152.9	83.0	3 °	34	16
	152.9	122.9	2 °	34	26
	152.9	137.9	1 °	34	18
IBMP	167.0	97.0	2 °	34	20
	167.0	125.0	1 °	34	16
SBMP	167.0	123.0	2 °	34	24
	167.0	138.0	1 °	34	18

4.3.5. Reversed phase separation of methoxypyrazines using optimized electrospray ionization conditions

As the sample extracts are expected to contain relatively large amounts of potentially interfering matrix elements (especially considering the concentration factor associated with the proposed sample preparation procedure), chromatographic separation is essential to separate the majority of the matrix elements from the peaks of interest. An optimized separation would involve gradient elution starting with a high aqueous fraction in the mobile phase. To compensate for the quenching effect of water experienced by early eluting peaks, the initial fraction of formic acid should correspondingly be increased. The three major wine methoxypyrazines, IPMP, IBMP and SBMP, are however strongly retained and may efficiently be eluted in 88% acetonitrile, where ionization efficiency is optimal. Alternatively, all five components may be determined with maximum efficiency by isocratic elution using the optimal mobile phase composition. As the mobile phase in the proposed isocratic separation contains a relatively high organic fraction, the reduced viscosity of the mobile phase provides the option to use longer columns to increase the separation efficiency.

Two possible strategies for the reversed phase separation of the components of interest were therefore identified for evaluation. These comprised gradient elution of the components of interest with post-column addition of optimized amounts of formic acid and isocratic elution at a mobile phase composition where electrospray ionization of the components of interest is optimal.

4.3.5.1. Gradient elution with post-column addition of optimized amounts of formic acid

The proportion of formic acid required to optimize electrospray ionization is relatively high and varies from approximately more than 10% in 50% aqueous acetonitrile to 2% in 95% aqueous acetonitrile. The relatively high amount of formic acid required in this range of mobile phase compositions precludes the use of the liquid chromatography system for its introduction as the silica-based analytical column can only tolerate a mobile phase pH of between 2 and 8.[8] The application of a gradient for the separation therefore demands that the required formic acid be added independently, via post-column addition. Formic acid was introduced via post-column addition utilizing a second LC system employing a time-programmed flow gradient to produce an optimal mobile phase composition throughout the separation. A relay switch on the primary separation system was used to synchronize the two LC systems. A pulse damper was utilized with the secondary liquid chromatography system, in an effort to produce pulse-free, consistent delivery of the post-column reagent.

The C18 column described in section 4.2.2. was used to perform the separation. The gradient used in the separation was as follows: 50% acetonitrile initially, increased to 90% acetonitrile in 5 minutes (via a concave curve), followed by 90% acetonitrile for 10 minutes, at a constant flow-rate of 0.4 mL/minute. Post-column addition of formic acid was done isocratically, by adding a 50% solution

in acetonitrile, via a flow-controlled gradient as follows: Starting at 0.1 mL/minute for 10.5 minutes, changing to 0.02 mL/minute in 30 seconds followed by 0.02 mL/minute for 3.5 minutes. The column temperature was 40°C for these experiments.

The method described above produced efficient separation of the components of interest. It was however found that the variation in peak area between injections was relatively large, with a relative standard deviation of approximately 20%. As the amount of formic acid in the mobile phase significantly affects the ionization efficiency, especially at relatively high aqueous compositions, small variations in the addition of formic acid to the mobile phase are expected to significantly affect the measured peak areas. It may reasonably be assumed that post-column addition of reagents would be more precise if the secondary system was used at higher flow-rates (due to pump limitations at these low flow-rates), but the addition of the post-column reagent was deliberately minimized to reduce the resulting peak-broadening.

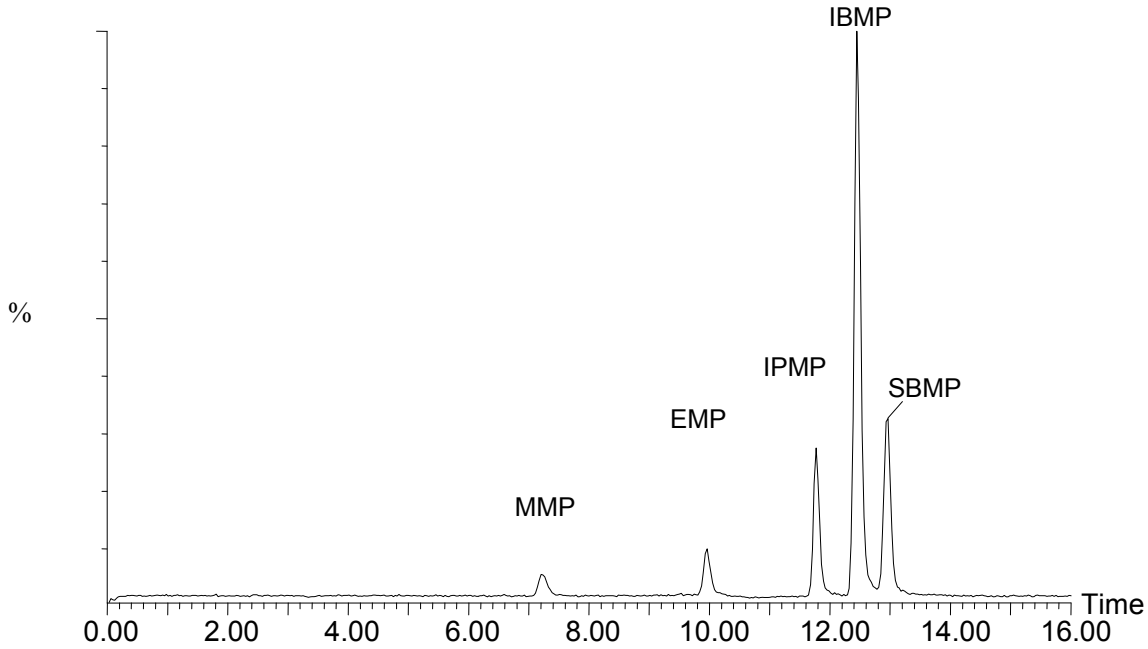


Figure 4.13.: Gradient reversed phase separation of a standard containing 20 pg MMP, EMP, IPMP and SBMP and 80 pg IBMP with post-column addition of optimized amounts of formic acid, ESI ionization.

Gradient separation as described produced efficient separation (Figure 4.13.) of the components of interest but as reproducibility of the results was poor the performance of the method was regarded as inadequate. Optimal method efficiency could not be realized utilizing gradient elution.

4.3.5.2. Isocratic elution at the optimal mobile phase composition

The optimal mobile phase composition for electrospray ionization of IBMP was determined to be 88% aqueous acetonitrile with 1% added formic acid. The pH of this solution is approximately 2.6, which is high enough for routine utilization with silica-based stationary phases. Isocratic reversed phase separation of the

components of interest with this mobile phase composition was therefore investigated.

As the composition of the mobile phase is dictated by the ionization efficiency, the only chromatographic parameter available for manipulation to achieve optimal separation (with the selected phase) is the efficiency, determined by the column length (and particle size). The column had a diameter of 4.6 mm with 3 µm particle size, the flow-rate was therefore maintained at 1.0 mL/minute while the column-oven temperature was kept at 40°C. Optimization experiments therefore basically reduced to the determination of the longest effective column that can be used at optimal conditions without generation of excessive back-pressure.

It was determined that a column of effective length of 300 mm produced efficient resolution of the components of interest. When operated with the specified mobile phase composition at 1.0 mL/minute and at 40°C, the back-pressure on the system did not exceed 152 bar. (The pressure limit of the column is 240 bar.)

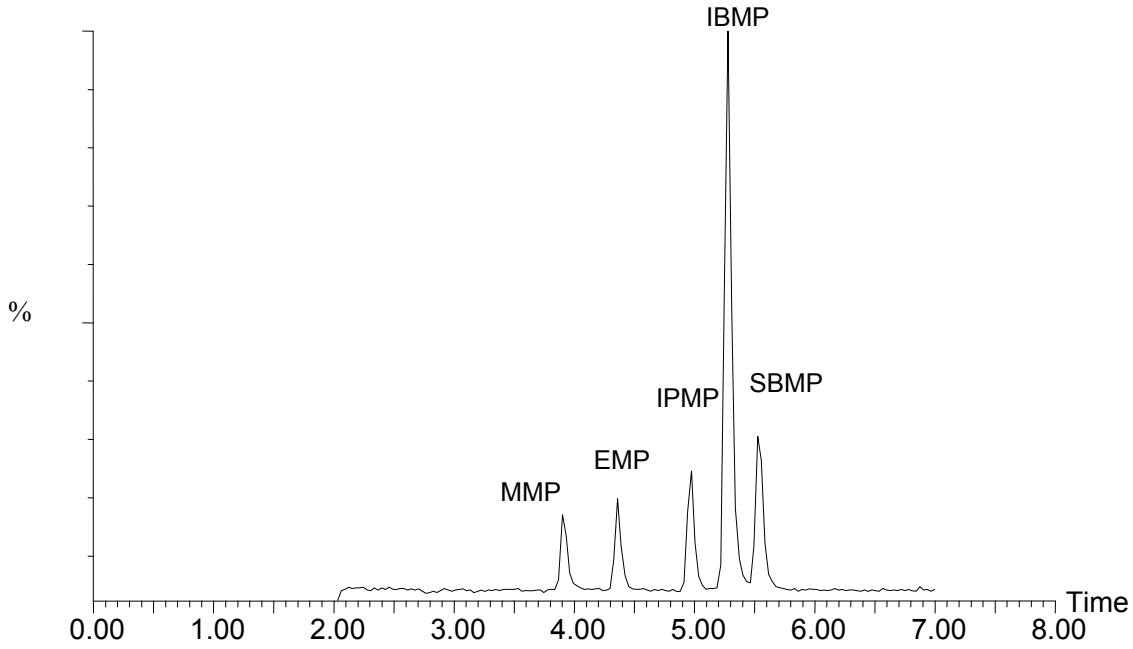


Figure 4.14.: Isocratic reversed phase separation of a standard containing 20 pg MMP, EMP, IPMP and SBMP and 80 pg IBMP, 300 mm C18 column (88% aqueous acetonitrile, 1% added formic acid), ESI ionization.

Isocratic elution utilizing an optimized mobile phase composition (88% acetonitrile, 1% formic acid) produced the highest ionization efficiency for all components of interest. Approximate detection limits were in the order of 0.1 pg on column for all five components. The reproducibility of results was good while the use of a column with effective length of 300 mm resulted in adequate resolution for all components. The six-port valve was utilized to divert the column effluent to waste prior to elution of the components of interest as well as thereafter. The effluent flow was switched to the detector only when the components of interest eluted to avoid fouling of the source with non-volatile sample matrix.

Isocratic elution, as described in this section, was therefore considered to be the most efficient mode of separation to be utilized in conjunction with electrospray ionization.

4.3.6. Method specificity utilizing isocratic reversed phase separation and electrospray ionization

A preliminary evaluation of method specificity revealed that the analyte response was attenuated by co-eluting matrix elements of the sample extract. It was concluded that electrospray ionization of the analyte are suppressed in the presence of concentrated matrix elements present in the extracts. Chromatographic separation, as described in paragraph 4.3.5.1. and 4.3.5.2., failed to resolve the analyte sufficiently from the interfering sample matrix to produce an unattenuated signal. Subsequent clean-up of the extracts with mixed mode reversed phase SPE, as described in Chapter 3 (3.3.4.2.2.), in combination with isocratic as well as gradient separation, similarly failed to resolve the interferons to affect levels of efficiency comparable to that obtained with the standards. ESI is not universally applicable to all analytes that can be ionized and it is widely accepted that some analytes are incompatible with this source.[4] Atmospheric pressure chemical ionization (APCI) is known to be less susceptible to matrix effects and was therefore subsequently evaluated for the analysis of 3-alkyl-2-methoxypyrazines in wine.

4.3.7. Atmospheric pressure chemical ionization

APCI was provisionally determined to be free from quenching effects caused by the sample matrix and was therefore optimized for possible utilization in the determination of methoxypyrazines in concentrated wine extracts. APCI can also

tolerate relatively high flow-rates and are therefore compatible with the use of 4.6 mm diameter separation columns operated at optimum flow-rates.[5]

4.3.8. Efficiency of atmospheric pressure chemical ionization in various elution systems utilized in reversed phase separations

The efficiency of APCI was evaluated in elution systems of aqueous acetonitrile and methanol. Sample introduction was by direct loop injections of a standard to produce 80 pg of analyte on column, with a mobile phase flow of 0.75 mL/minute. The quaternary pump was utilized to produce various mobile phase compositions starting from 50% organic phase and increasing to 98% in 10% increments. A constant composition of 1% formic acid was used in each of these mobile phases, as this was found to provide optimal ionization efficiency in ESI. The MRM transitions indicated previously for ESI were used to evaluate the effect of the mobile phase composition. The results are summarized in Figure 4.15.

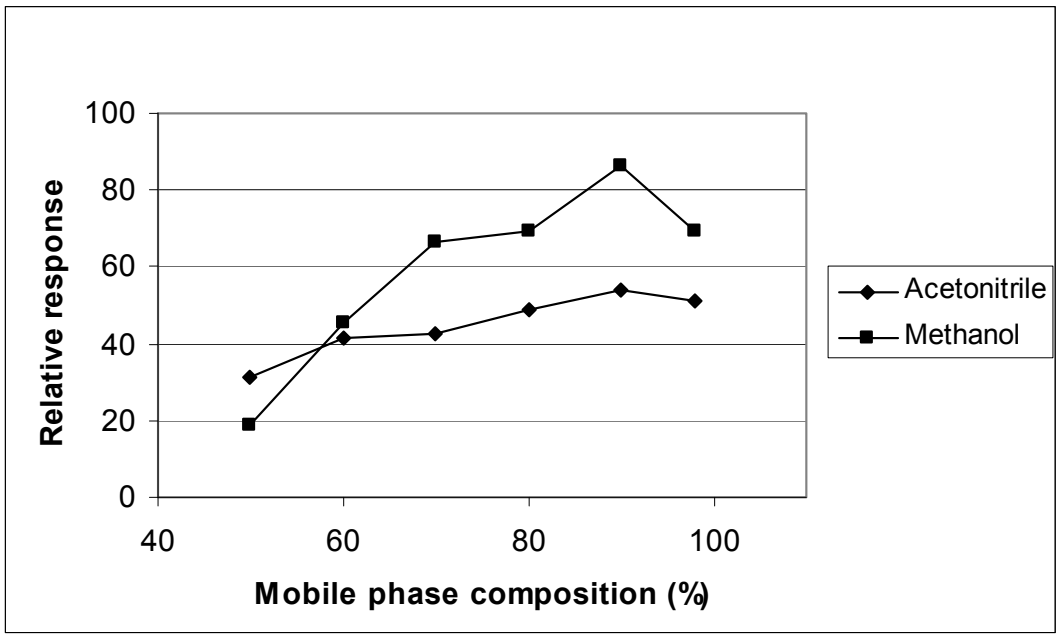


Figure 4.15.: APCI ionization efficiency of IBMP in acetonitrile- and methanol based mobile phases, with 1% formic acid.

APCI efficiency in the aqueous methanol system (1% formic acid) was found to produce higher sensitivity (above 60% organic phase).

Protonation of the target species is a premise for efficient positive mode APCI, and as a volatile acidic modifier was found to significantly affect ESI ionization efficiency, the amount of formic acid in the mobile phase was further optimized for APCI. Sample introduction and detection was performed as outlined above while mobile phase compositions ranged from 0 to 2% formic acid in 90% aqueous methanol and acetonitrile respectively. The results for the MRM acquisitions for IBMP are summarized in Figure 4.16.

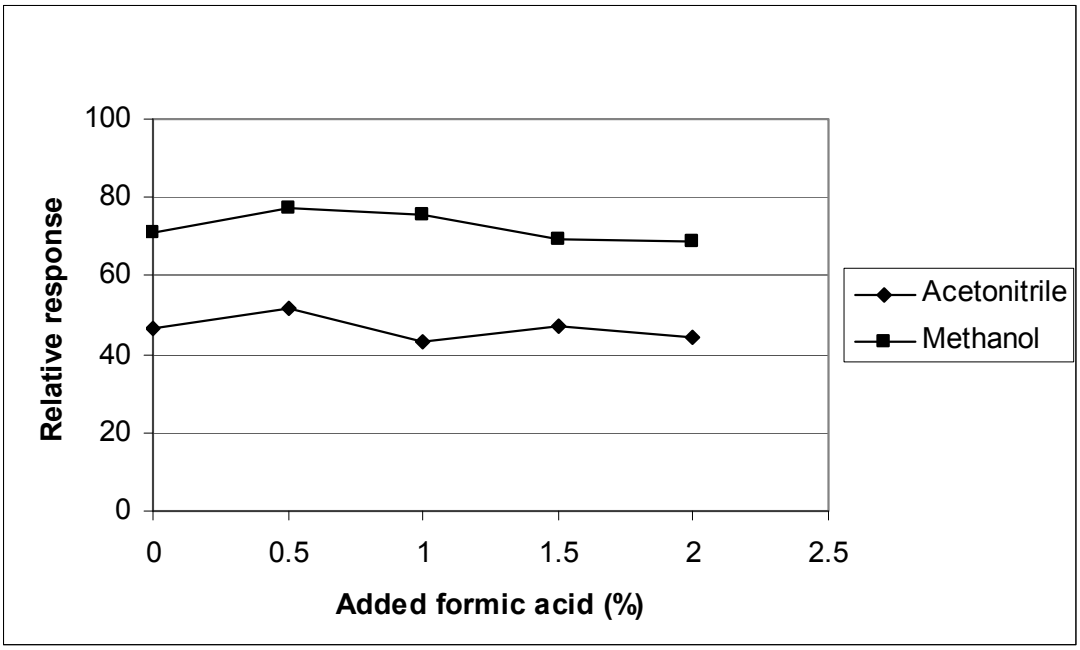


Figure 4.16.: Ionization efficiency in 90% aqueous acetonitrile and methanol mobile phases with various amounts of added formic acid.

Formic acid, added to an effective concentration of 0.5% was found to be optimal in both mobile phase systems. The improvement in ionization efficiency realized with the use of added formic acid (up to 2%) was insignificant, utilization of this reagent was therefore suspend. It is also evident that ionization in aqueous

methanol is more efficient throughout the range investigated, and methanol was therefore selected as the organic modifier in the reversed phase separations performed with APCI.

4.3.9. Effect of the orientation of the APCI probe on ionization efficiency

In the APCI interface, the mobile phase stream enters the probe where it is pneumatically converted to an aerosol, which is then rapidly dried and converted to the gas phase. The position of the probe may be adjusted parallel to the axis running through the ion optics and the pinhole entrance to the mass spectrometer. The effect of this adjustment is to orientate the spray optimally relative to the corona discharge needle and the sampling cone. The position of the spray relative to the corona discharge needle and the sampling cone is critical for the respective processes of ionization and ion transfer. The source is equipped with a vernier probe adjuster to facilitate precise positioning of the tip.

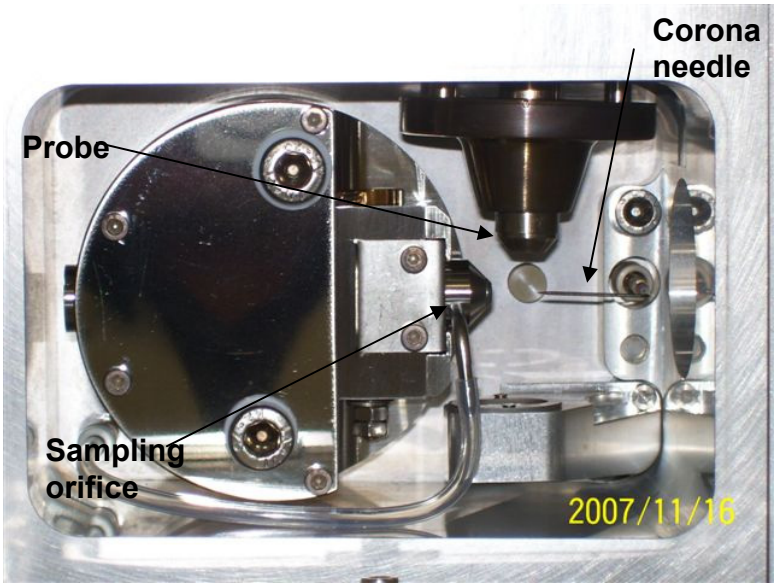


Figure 4.17.: APCI source illustrating the position of the probe, corona needle and sampling orifice.

The effect of probe orientation on the APCI efficiency was evaluated by making repeated direct loop injections of a standard to produce 80 pg of analyte on column in conjunction with a mobile phase flow of 0.75 mL/minute, while adjusting the probe through the range of possible settings. MRM transition signals were used to observe the effect of the probe orientation. As the position of the APCI probe was found to have a significant effect on the efficiency, further experiments were also carried out at intermediate vernier settings (240 pg on column) thus refining the optimal probe position. The results for IBMP in the range of possible probe orientations are presented in Figure 4.18.

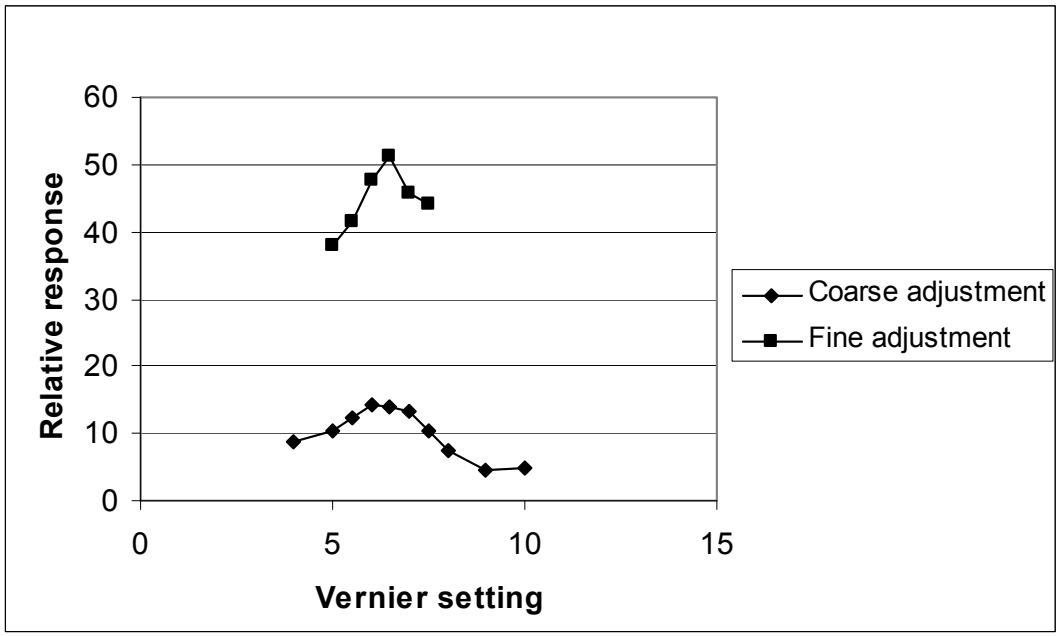


Figure 4.18.: Effect of probe orientation on ionization efficiency of IBMP.

Optimized ionization and introduction of ions is achieved when the effluent spray is directed approximately at the mid-point between the corona discharge needle and the sample cone. The vernier setting corresponding to the best signal was 6.5.

4.3.10. Effect of mobile phase flow-rate on ionization efficiency

The effect of mobile phase flow-rate on APCI ionization efficiency was evaluated by observing the intensity of the signal obtained while varying the flow from the liquid chromatograph between 0.2 and 1.1 mL/minute. The mobile phase composition was 90% methanol with 1% formic acid. The response for IBMP as a function of flow-rate is presented below (Figure 4.19.).

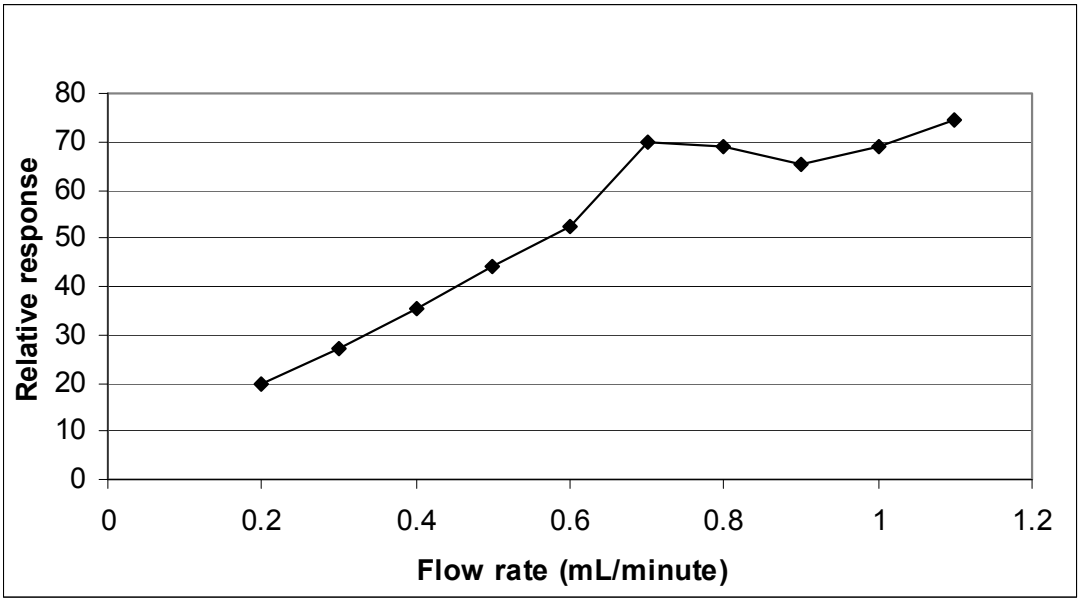


Figure 4.19.: APCI ionization efficiency of IBMP at various mobile phase flow-rates.

From these data it can be concluded that the efficiency essentially increases with flow rate across the studied range. Higher flow-rates appears to enhance the efficiency, a phenomenon that that may be explained by the fact that in APCI analyte molecules are ionized by interaction with ionized solvent molecules so that at higher flow-rates more interactions, and hence higher efficiencies, are possible.[4] This suggests that a 4.6 mm diameter analytical separation column and flow rates close to or at the optimal flow rate of 1.0 mL/minute may be used in the current application.

4.3.11. Optimisation of APCI source parameters

The corona discharge current may have a significant effect on sensitivity and depends upon the polarity of the compound as well as the analytical mobile phase.[9] The optimal corona discharge current was determined utilizing the same protocol as before and observing the effect of the corona discharge current on the intensity of the signals obtained (Figure 4.20.). A corona discharge current of 4.4 μA was found to provide the best signal response and was therefore used in subsequent experiments.

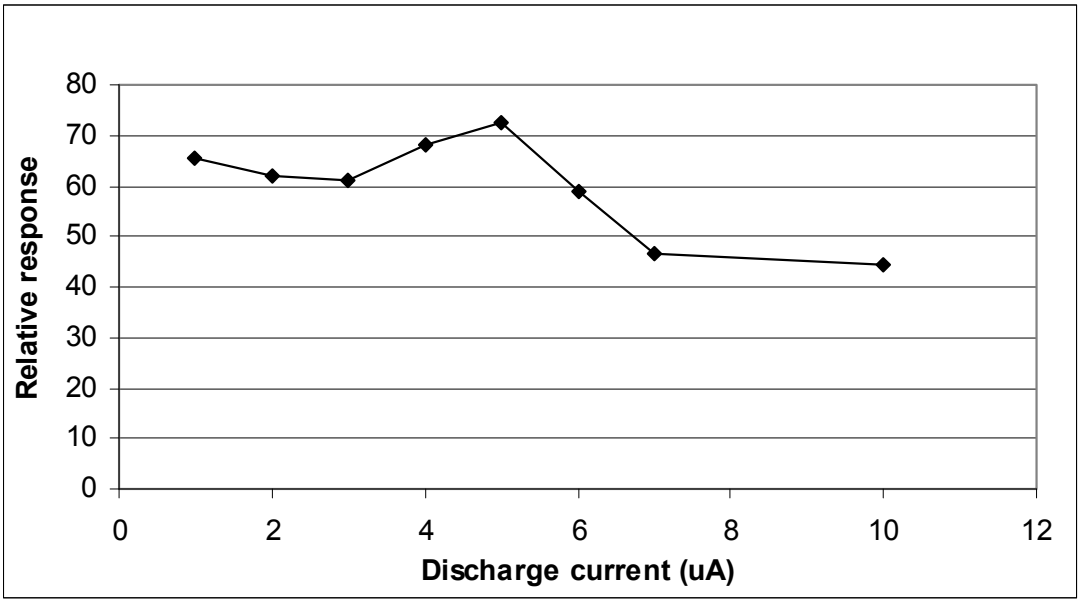


Figure 4.20.: APCI ionization efficiency at various corona discharge currents.

Inside the APCI probe, the eluent stream emerging from the fused silica capillary is converted to an aerosol spray of fine droplets. The nebulizer plume is then rapidly heated in a stream of nitrogen gas to ensure that it leaves the probe as a stream of desolvated sample droplets. The support gas controls the droplet residence time and allows the sample to be swept from the probe efficiently.[9]

The desolvation gas flow was optimized by observing the effect of varying desolvation gas flow on the APCI efficiency. Figure 4.21. depicts the effect of gas flow on the ionization efficiency.

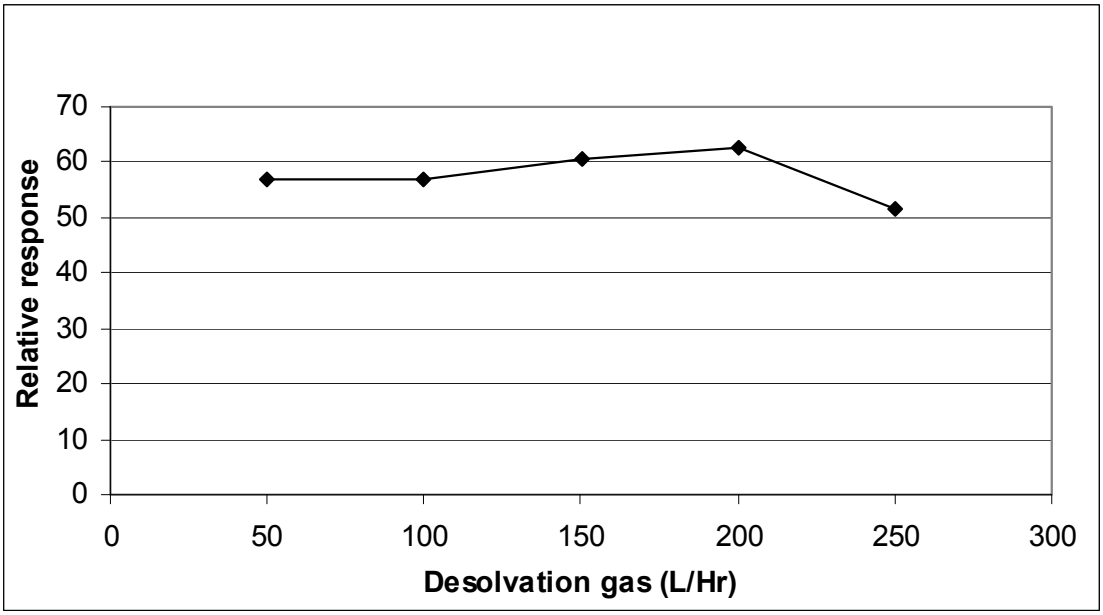


Figure 4.21.: APCI efficiency for IBMP at various desolvation gas flows.

The desolvation gas flow had a relatively small effect on the signal intensity. The manufacturer nevertheless recommends that optimization be performed to minimize chemical background noise levels. A flow of 200 L/Hr was selected as optimal for subsequent analysis as a slight increase in signal was observed at this flow. Altering the flow of support gas similarly had little effect on the intensity of the signal obtained hence a setting of 1, as is recommended by the manufacturer, was accepted for subsequent experiments.[9]

The aerosol spray of fine droplets emerging from the fused silica capillary is heated inside the APCI probe so that the combination of heat and desolvation gas flow of may desolvate the sample droplets. The APCI probe temperature

may be controlled to ensure efficient desolvation of the analyte droplets without possible thermal decomposition of the target species.[9]

The probe temperature was optimized by observing the intensity of signals obtained for different desolvation temperatures while maintaining the desolvation gas flow at the previously determined optimal value of 200 L/hour.

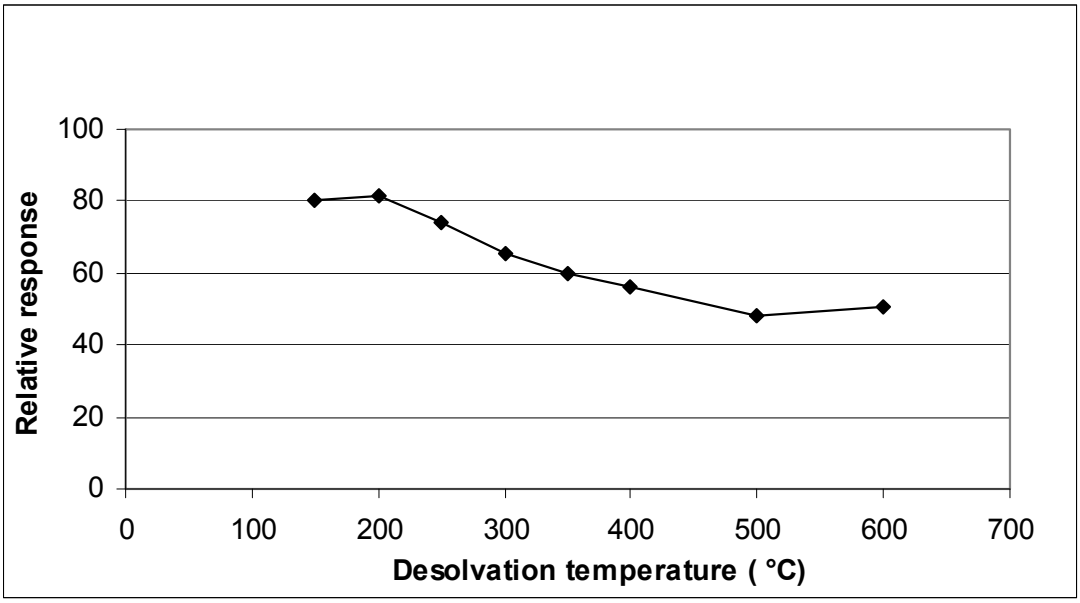


Figure 4.22.: APCI efficiency of IBMP at various desolvation temperatures at a desolvation gas-flow of 200 L/hour.

A desolvation temperature of 200°C was determined to be optimal (Figure 4.22.). The maximum desolvation temperature recommended for operation of the APCI probe is 650°C.[9] The observed optimal temperature is therefore relatively low, a phenomenon that may be ascribed to the fact that the analyte molecules are relatively volatile and that the eluting mobile phase is similarly volatile. Optimal efficiency may therefore simply be achieved at relatively low desolvation temperatures.

4.3.12. Optimisation of instrument tuning parameters for MS-MS operation

For optimal quantitative MRM analysis, the MS tuning parameters were adjusted to achieve the highest sensitivity for each analyte. Tuning was performed by infusing 5 mg/L standards of the methoxypyrazines under investigation at 15 µL/minute via a syringe pump, in conjunction with a flow of 1.0 mL/minute from the liquid chromatograph, at the mobile phase composition encountered in the separation (80% aqueous methanol). The parameters were optimized by observing the intensity of the relevant ions while experimental parameters were adjusted (Table 4.7.). The inter channel delay time and the inter scan time was 0.020 seconds and 0.100 seconds respectively.

Table 4.7.: Tuning parameters for APCI MS-MS operation.

Parameter	Value
Source: IonSABRE APCI source, positive mode	
Corona (µA)	4.4
Cone (V)	34
Extractor (V)	6.00
RF Lens (V)	0
Source Temperature (°C)	150
Desolvation Temperature (°C)	200
Cone Gas Flow (L/Hr)	50
Desolvation Gas Flow (L/Hr)	200
Analyzer	
LM 1 Resolution	10.0
HM 1 Resolution	10.0
Ion Energy 1	0.3
Entrance	-5
Collision (eV)	18
Exit	0
LM 2 Resolution	14.0
HM 2 Resolution	14.0
Ion Energy 2	1.0
Multiplier (V)	700
Collision Gas Flow (L/Hr)	0.35

Table 4.8.: MRM transitions used.

Compound	Parent Ion (Da)	Daughter Ion (Da)	Transition	Cone (V)	Collision (eV)
MMP	125.0	56.2	2 °	34	20
	125.0	97.0	1 °	34	14
EMP	139.0	111.0	2 °	34	16
	139.0	123.9	1 °	34	18
IPMP	152.9	122.9	2 °	34	26
	152.9	137.9	1 °	34	18
IBMP	167.0	124.0	2 °	34	22
	167.0	125.0	1 °	34	16
SBMP	167.0	123.0	2 °	34	24
	167.0	138.0	1 °	34	18

The fragmentation patterns observed for the ion transitions listed in Table 4.8. correspond mainly to cleavage of the alkyl side-chain at the alkyl-substituted carbon atoms. The observed cleavage of the branched alkyl side-chains are due to charge stabilization at the position of branching. Generally the largest substituent at a branch is eliminated most readily (SBMP, 167 > 138). In alkyl-substituted aromatic compounds, cleavage is also very probable at the bond β to the ring (IPMP, IBMP and SBMP). The aromatic ring stabilizes resulting cationic daughter ion by charge delocatization.[10] The relevant mass spectra obtained are attached as Appendix 1. Table 4.9. presents accurate mass information and proposed molecular formulae pertaining to the molecular ions and daughter ion fragments listed in Table 4.8. Accurate mass determinations were performed utilizing the Q-TOF instrument specified in the experimental section (4.2.2.). The integrity of the data is evident from the close correlation between the observed and theoretical masses obtained for the relevant molecular ions (M + 1). Furthermore it can be noted that the observed masses of the primary and secondary daughter ions correlate very well with the theoretical masses of the proposed molecular formulae indicating a high degree of confidence in the proposed elemental compositions of these secondary ions.

Table 4.9.: Accurate mass information and molecular formulae pertaining to ion transitions of interest.

Compound	M + 1 Observed mass (Theoretical mass) (Da)	Daughter 1 Observed mass (Da)	Elemental composition (Theoretical mass) (Da)	Daughter 2 Observed mass (Da)	Elemental composition (Theoretical mass) (Da)
MMP	125.0721 (125.0715)	97.0765	$C_4H_5N_2O$ (97.0402)	56.0497	C_2H_2NO (56.0136)
EMP	139.0873 (139.0871)	124.0656	$C_6H_8N_2O$ (124.0637)	111.0910	$C_5H_7N_2O$ (111.0558)
IPMP	153.1028 (153.1004)	138.0806	$C_7H_{10}N_2O$ (138.0793)	123.0567	$C_6H_7N_2O$ (123.0558)
IBMP	167.1184 (167.1160)	125.0714	$C_6H_9N_2O$ (125.0715)	124.0639	$C_6H_8N_2O$ (124.0637)
SBMP	167.1180 (167.1160)	138.0783	$C_7H_{10}N_2O$ (138.0793)	123.0572	$C_6H_7N_2O$ (123.0558)

4.3.13. Method specificity utilizing separation on a C18 column and atmospheric pressure chemical ionization

A preliminary evaluation of method specificity revealed that the analyte response for IBMP and SBMP was not affected by the matrix of the extracts and that the peaks of interest were sufficiently resolved from interferences. An unknown interfering compound, with identical molar mass and daughter ions, was however found to co-elute with IPMP. Co-elution was evident from the fact that the ion ratio for the two transitions were not consistent with that obtained for the calibration standards. Identification of the interferon was attempted utilizing off-line GC-MS and LC-TOF analysis of collected fractions but was unsuccessful. Lacey *et al.* reported similarly that matrix elements interfered with the determination of IPMP in Sauvignon blanc wines with GC-MS.[11] Separation of the interferon from IPMP was attempted by additional optimization of the solvent gradient. Changes in the solvent gradient failed to achieve separation of the interferon from IPMP. As IPMP is, after IBMP, probably the most abundant 3-alkyl-2-methoxypyrazine in Sauvignon blanc wine[11], the relevant quantitative

information may be essential in the characterization of the methoxypyrazine profile of the cultivar and detection of adulteration of the wine. It is therefore imperative that this compound be separated from interferences so that it may be quantified successfully.

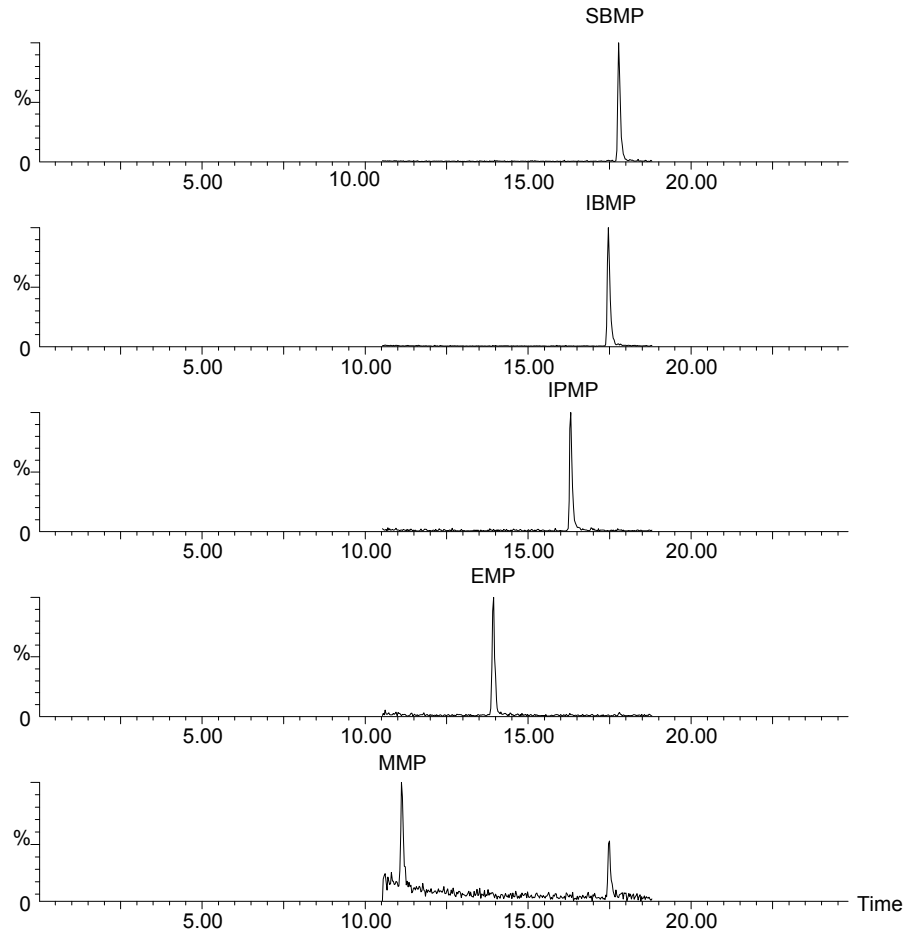


Figure 4.23.: Standard of MMP (68.4 pg), EMP (65.9 pg), IPMP (61.5 pg), IBMP (238.4 pg) and SBMP (60.4 pg), separated on C18 column.

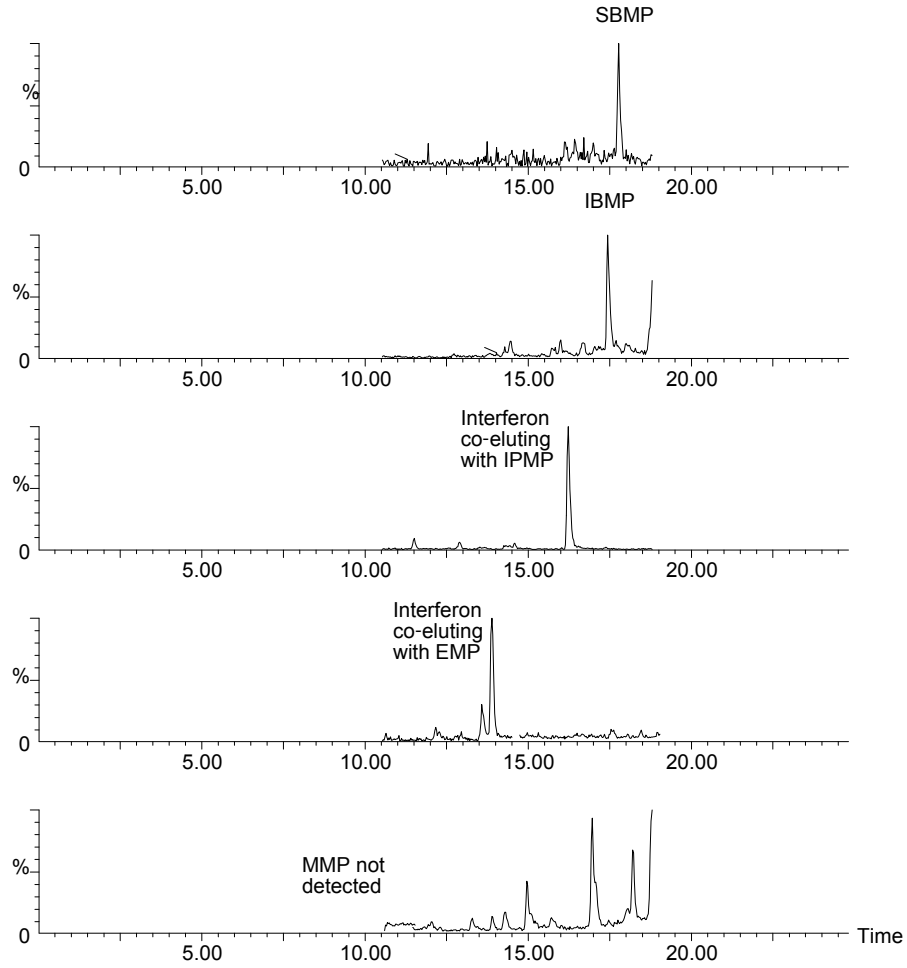


Figure 4.24.: Sample extract separated on C18 column showing co-eluting compounds.

Three separate strategies were then evaluated for resolution of IPMP from the interferon, namely SPE clean-up of the extract, utilization of alternative column chemistries to achieve sufficient selectivity to affect separation and online sample clean-up and separation utilizing a secondary column in tandem with the primary separation column.

4.3.13.1. SPE clean-up of extracts for reversed phase separation on a C18 column

Sample clean-up utilizing Oasis mixed mechanisms SPE in combination with distillation and solvent extraction, as described in Chapter 3 (3.3.4.2.2.), was evaluated to remove the interfering compound co-eluting with IPMP. Oasis SPE cartridges contain a sorbent that is a copolymer composed of divinylbenzene and N-vinylpyrrolidone units and is designed to have a hydrophilic-lipophilic balance when used under reversed phase conditions. This procedure was optimized to enhance retention of basic compounds and it was hoped that an alternative selectivity would be achieved with this method so that the interferon might be removed. It was however found that the co-eluting interferon persisted upon separation of the purified extract on a C18 column. Quantification of IPMP was therefore not possible utilizing this method with additional SPE sample clean-up.

4.3.13.2. Evaluation of alternative column chemistries to achieve improved selectivity for the reversed phase separation of the 3-alkyl-2-methoxypyrazines

Alternative reversed phase column chemistries were evaluated for the separation of sample extracts to ensure resolution of analytes from interfering matrix elements. These experiments were also aimed at identifying column chemistries that may be utilized in tandem for on-line sample clean-up and separation, described in the following section. Additional column chemistries that were evaluated were phenyl-hexyl, C8, C5 and cyano (column details in Table 4.1.).

It was observed that the phenyl-hexyl as well as the C8 columns provided separation of the three major 3-alkyl-2-methoxypyrazines (IPMP, IBMP and SBMP) from major matrix interferences. However, major matrix interferences co-eluted with the minor 3-alkyl-2-methoxypyrazines (MMP and EMP). The phenyl-

hexyl column produced stronger retention of the analytes which afforded elution with a mobile phase containing a relatively high fraction of volatile organic solvent. The gradient used for elution of the analytes from the phenyl-hexyl phase afforded elution of the major 3-alkyl-2-meyhoxypyrazines in a mobile phase consisting of approximately 80% methanol, while similar elution on the C8 phase was achieved in approximately 70% methanol. The advantages of eluting the analytes in a mobile phase that contains a relatively high fraction of volatile solvent are more efficient solvent removal in the ionization source and hence better sensitivity as well as potential separation from interferences, as discussed earlier.

4.3.13.3. Online clean-up of extracts utilizing a secondary column in tandem with the primary separation column

The LC-MS system incorporates a six-port valve that enables the system to be configured for sample injection on one column (preparative or secondary column) followed by time-programmable switching of the effluent to the main separation (or primary) column. A premise for successful reversed phase on-line sample clean-up and separation utilizing a secondary column in tandem with the primary separation column, is that the organic content of the eluting mobile phase on the secondary column must not exceed that of the starting mobile phase on the primary separation column, to facilitate concentration of the solute at the head of this column. The preferred column combination for such a strategy would therefore require that the compounds of interest be eluted from the secondary column utilizing a mobile phase that has a relatively high aqueous content while separation on the primary column is achieved with a mobile phase with high organic content. The cyano column (50 x 4.6 mm, 5 µm) was selected as the secondary column and phenyl-hexyl (250 x 4.6 mm, 5 µm) as the primary separation column, as they are relatively polar and apolar respectively and correspondingly required low and high organic content of the mobile phase for

elution of the compounds of interest. It is also expected that relatively apolar interferences may efficiently be removed from the analytes by separation on the relatively polar secondary column. Separation on the apolar primary column may then possibly be free from strongly retained interferences that co-elute with the substances under investigation.

The separation was performed as follows: The sample extract was injected on the secondary column and the effluent diverted to waste. The solvent program started at 5% methanol, constant for 1 minute, increasing to 40% methanol in 7 minutes via a linear gradient followed by 40% methanol for 1 minute. At 7 minutes the effluent was switched from waste to the primary separation column. At 8 minutes the methanol content was increased linearly from 40% to 90% in 12 minutes. After 1 minute, the methanol content was decreased to 15% (equilibration of primary separation column). The effluent was diverted to waste at run-time 26 minutes upon which the secondary column was equilibrated at 5% methanol for 1.5 minutes, for a total run-time of 27.5 minutes. The flow was constant throughout the separation at 1.0 mL/minute and the column-oven temperature was controlled at 40°C. The maximum pressure did not exceed 160 bar.

It was found that separation on the two-column system described affected separation of the major interferences from IPMP. Co-eluting matrix elements were however still found to interfere with the determination of the minor 3-alkyl-2-methoxypyrazines (MMP and EMP). Figure 4.25. presents a chromatogram obtained for a standard that was separated utilizing the described method. It can also be noted that MMP is not detected at this concentration. This compound eluted in a relatively aqueous mobile phase, and ionization parameters (especially desolvation temperature of 200°C) was optimized for ionization of methoxypyrazines in mobile phases that contains a higher organic content. Ionization efficiency in the relatively aqueous phase was therefore not optimal and may account for the absence of MMP from the chromatogram.

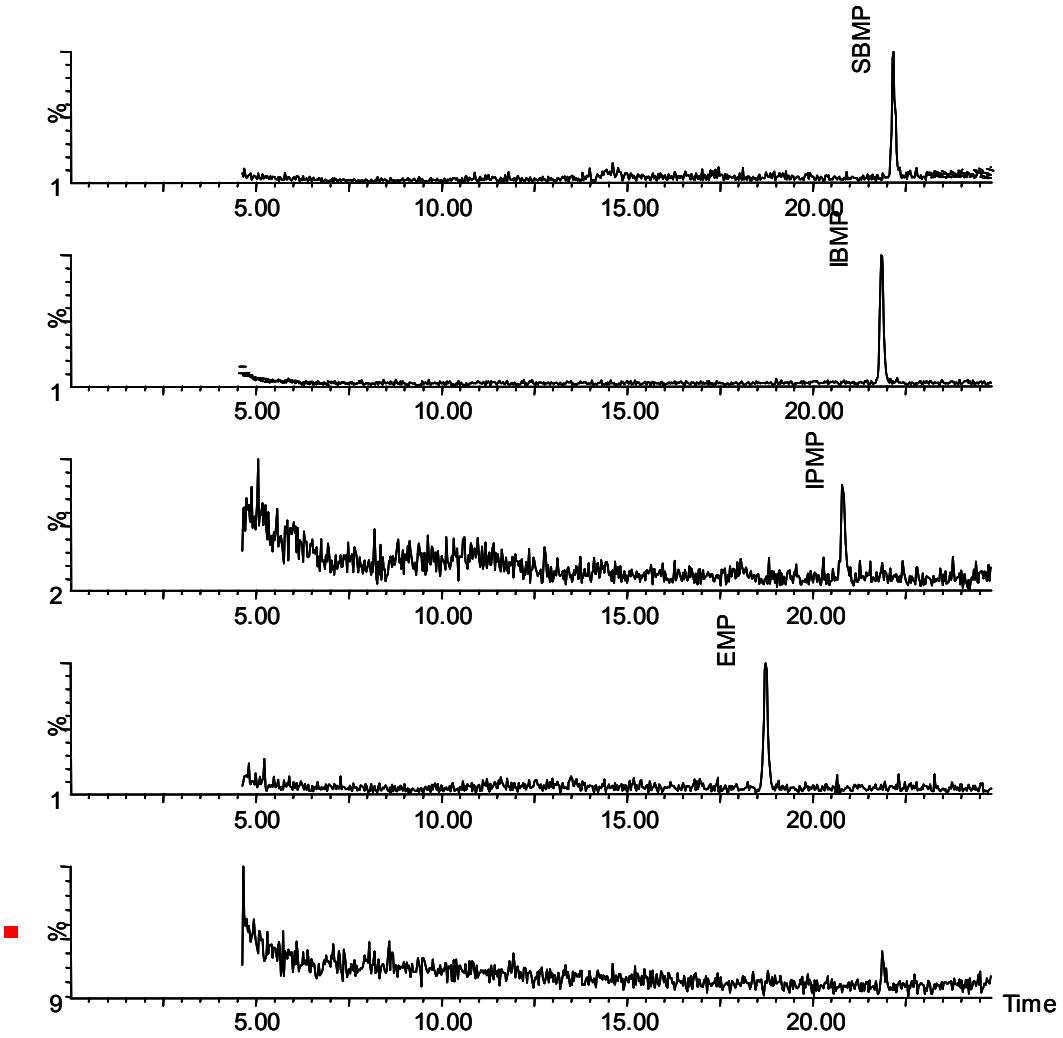


Figure 4.25.: Chromatogram of standard solution containing 13.7 pg MMP, 13.2 pg EMP, 12.3 pg IPMP, 47.6 pg IBMP and 12.1 pg SBMP. Reversed phase separation utilizing CN functionalized secondary column in tandem with Ph-hexyl primary separation column.

4.3.14. LC-APCI-MS analysis of methoxypyrazines in wine

In summary, it was shown that APCI is more efficient utilizing methanol as the mobile phase organic modifier, and that ionization efficiency generally increases

with methanol content and the addition of formic acid (up to 1%). Since optimal sensitivity depends on the amount of methanol in the mobile phase, the chromatographic separations of methoxypyrazines were optimized to achieve separation via gradient elution in order to ensure elution of the target analytes with a methanol-rich mobile phase. As the addition of formic acid affected a relatively small increase in efficiency it was not used in the method due to the effect it may have on the longer term stability of the silica-based separation columns utilized. The two-column system as well as the phenyl-hexyl column affected resolution of the three important 3-alkyl-2-methoxypyrazines (IPMP, IBMP and SBMP) from most major matrix interferences. It was finally decided that the phenyl-hexyl phase provides the highest efficiency for separation of the sample extracts and as the procedure is relatively uncomplicated compared to the former, it was accepted for utilization in the investigation. A preliminary evaluation of method specificity revealed the analyte response was not affected by the matrix of the extracts and that the peaks of interest were sufficiently resolved from major interferences (Figure 4.27.). The two minor 3-alkyl-2-methoxypyrazines, MMP and EMP, were however completely obscured by unresolved matrix elements. Matrix interference was observed to be more problematic with the said congeners for two reasons. Firstly they have lower molecular ion-and daughter-ion masses, interfering ions were therefore possibly more common. Secondly these were also retained less strongly by the stationary phase not only resulting in elution in a region of the chromatogram where most of the sample matrix is expected, but also eluting in a relatively aqueous fraction of the mobile phase, thereby reducing their detectability. As these compounds were also expected to have very low concentrations and are not known to be present or to play a role in the odor of the cultivar, they were excluded from the investigation. The focus was therefore exclusively on the major 3-alkyl-2-methoxypyrazines (IPMP, IBMP and SBMP) from this point onwards.

As separation of closely eluting compounds is critical in this application, the efficiency of the designated column must be optimal. The column dimensions

was limited to 250 x 4.6 mm with particle size of 5 µm to achieve high relative efficiency (longer column) while limiting the backpressure (larger particle size) resulting from separation via a methanol and water gradient. The optimized gradient separation utilizing the phenyl-hexyl phase was as follows: The gradient started at 35% methanol, increased (linearly) to 85% methanol in 18 minutes followed by column clean-up at 95% methanol for 2.5 minutes. The flow was maintained at 1.0 mL/minute throughout and re-equilibration time was 3.5 minutes. The column temperature was maintained at 40°C and the total run-time was 25 minutes while the divert valve was used to direct the effluent to the detector only between time 14 and 18 minutes, the rest going to waste. Figure 4.26. and 4.27. represents MRM chromatograms obtained for a standard and wine extract utilizing separation on this phase (only primary ion transitions shown).

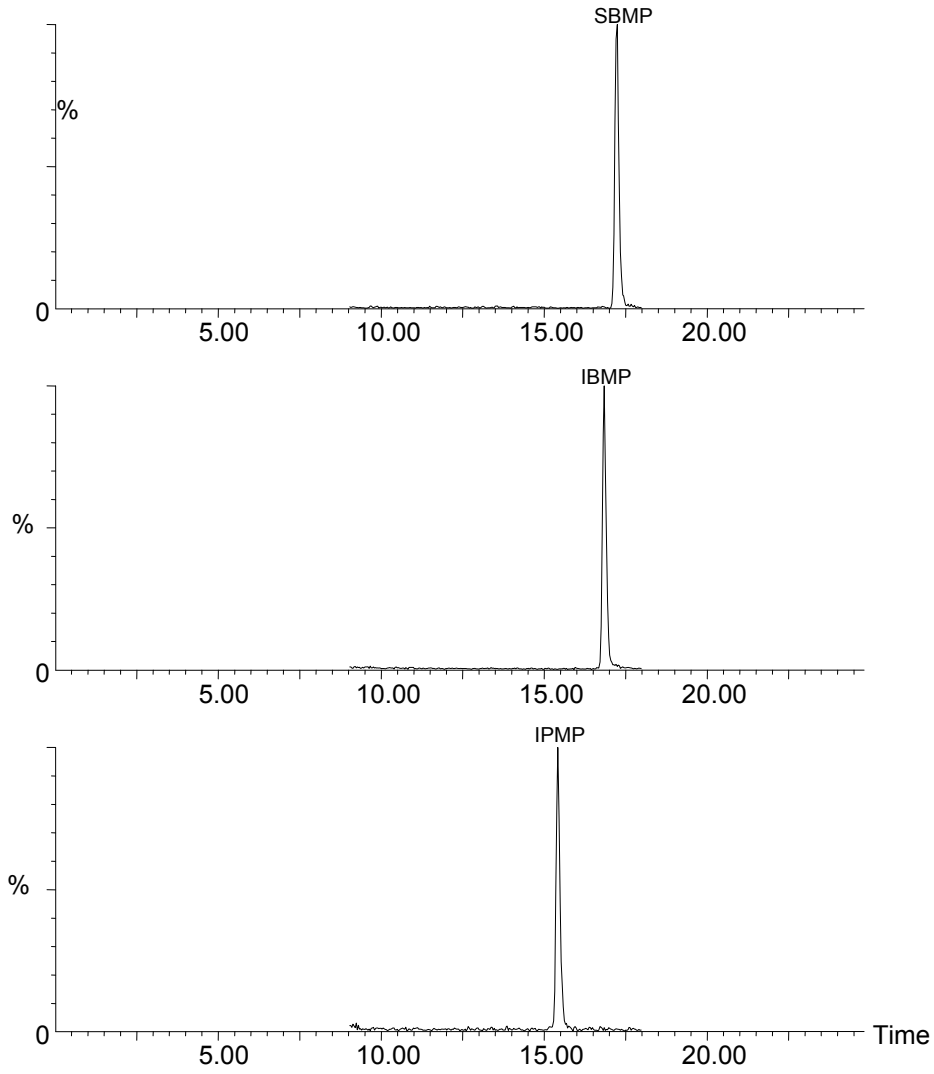


Figure 4.26: Chromatogram of a standard containing 61.6 pg IPMP, 238.4 pg IBMP and 60.4 pg SBMP. Gradient separation on phenyl-hexyl column.

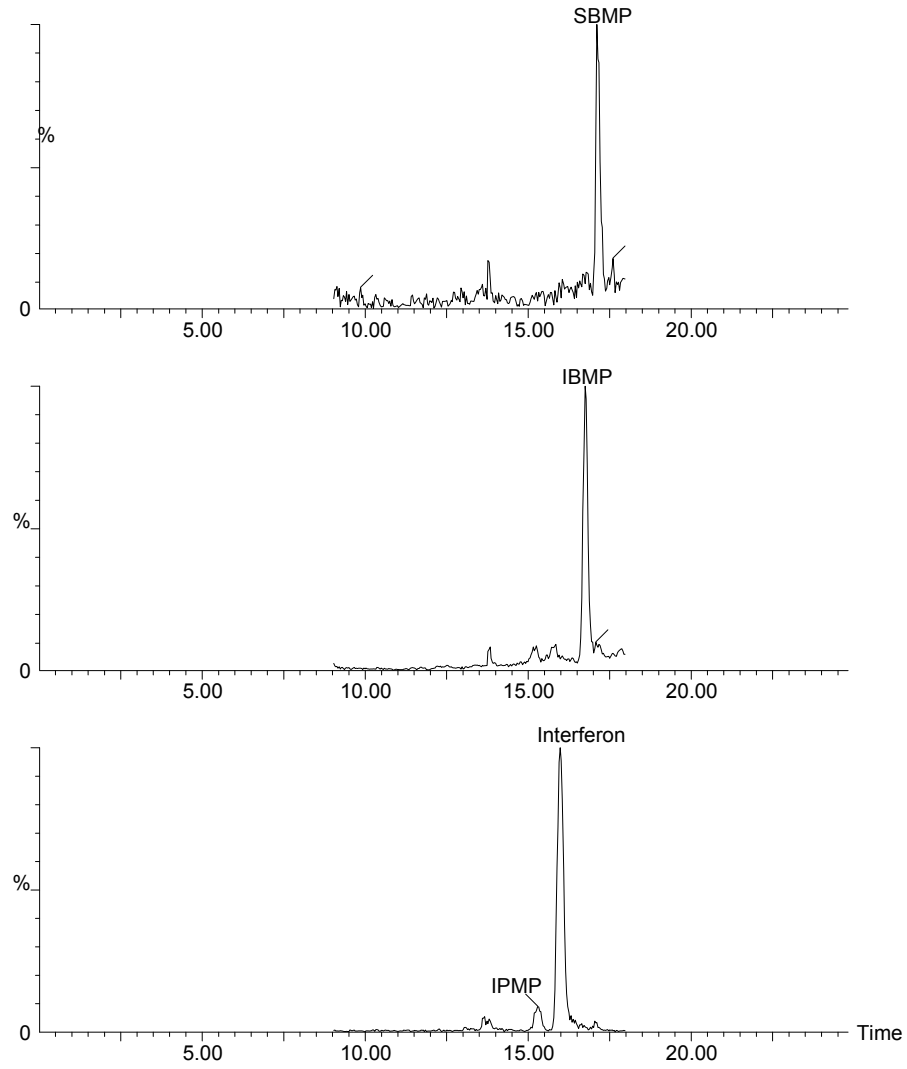


Figure 4.27: Chromatogram of a Sauvignon blanc wine extract, separated on a phenyl-hexyl column using the optimized separation conditions (100 µL injected). Extract prepared as described in experimental section (concentration factor 500). Interferon separated from IPMP peak.

4.4. Conclusions

Electrospray ionization was utilized initially for ionization of five 3-alkyl-2-methoxypyrazines. The mobile phase and tuning parameters were optimized

using standards and relatively high efficiencies were obtained. It was however subsequently found that the matrix of the wine extracts caused severe quenching of the ionization and that no form of additional sample clean-up, separation or tuning could resolve this defect.

ESI generally requires low mobile phase flow-rates.[4] The manufacturer of the system claims that flow-rates of up to 1.0 mL/minute may be accommodated but that typical values ranging between 0.1 and 0.2 mL/minute are preffered.[6] It has however been reported that (low) flow-rate may be critical for efficient ESI (depending on various factors including instrument design) and it is possible that the flow-rate utilized with ESI in this investigation (1.0 mL/minute) was too high and that this contributed to the quenching effects that were experienced in the presence of the wine matrix.[12] The flow-rate used (1.0 mL/minute) was optimal for the 4.6 mm diameter separation columns while flow splitting was not considered due to the sensitivity requirements of the method. The larger diameter columns (4.6 mm) were utilized as opposed to that of 2.1 mm as the former provides larger loading capacity[8], a critical factor for efficient separation of concentrated extracts via large injection volumes. It was also determined that with standards, the efficiency of ESI did not decrease with flow-rates between 0.2 and 0.7 mL/minute and that it was relatively unchanged between 0.7 and 1.0 mL/minute. With real samples containing extremely concentrated matrices, the flow-rate requirements for optimal ionization efficiency might have been different from those attainable with standards.

Atmospheric pressure chemical ionization was then optimized for use with the reversed phase separation method utilizing a C18 phase. Sensitivity obtained with APCI was approximately an order of magnitude lower than with ESI but ionization of the analytes were unaffected by the wine sample matrix. It was then found that unknown matrix components co-eluted with IPMP as well as MMP and EMP. Separation selectivity was improved by utilizing a phenyl-hexyl separation phase to resolve IPMP while MMP and EMP was omitted from the investigation.

In conclusion, ESI and APCI were evaluated for the trace-level analysis of 3-alkyl-2-methoxypyrazines in wine. ESI is more efficient, but was not suitable due to matrix effects. APCI avoided this problem, and an optimized method for the analysis of the three major wine 3-alkyl-2-methoxypyrazines was developed which produced good performance. This optimized APCI method with separation on a phenyl-hexyl phase was subsequently validated (Chapter 5) for the determination of three 3-alkyl-2-methoxypyrazines (IPMP, IBMP and SBMP) in South African Sauvignon blanc wine.

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CHAPTER 5

Validation of the optimized RP-LC-APCI-MS method for the analysis of 3-alkyl-2-methoxypyrazines in wine

5.1. Introduction and objectives

The objective of this study is in the first instance to quantify selected 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine and secondly to use the information to characterize authentic wine in this regard. In this Chapter the performance characteristics of the analytical method developed previously will be validated to illustrate that the method is capable of producing the required quantitative analytical data.

5.2. Materials and methods

5.2.1. Chemicals and samples

Acetonitrile, dichloromethane and tannic acid were from Merck (Darmstadt, Germany), IBMP, IPMP, SBMP, methanol and ethanol from Aldrich (Sigma-Aldrich, South Africa) and formic acid from Associated Chemical Enterprises (ACE, South Africa).

Standards were prepared by weighing appropriate amounts of reference material on an analytical balance followed by dilution utilizing A-grade volumetric glassware. Intermediate standards were prepared in ethanol while working standards were prepared in 10% ethanol.

Wine samples for the recovery study were prepared by mixing together at random, various bottled Sauvignon blanc wines to establish batches of at least 5 L. Aliquots of each batch were then transferred to 1 L volumetric flasks, to which appropriate amounts of standard were added and finally made up to the mark with wine, to produce series of fortified wine samples. Each series of wine samples thus consisted of unfortified wine as well as fortified wine (three levels of fortification: 1, 10 and 100 ng/L).

5.2.2. Sample preparation

Aliquots of wine samples were transferred to an A-grade 500 mL volumetric flask up to the mark. The contents of the flask were then transferred quantitatively to a 1 L boiling flask by repeated rinsing with water. After a single glass ball and spatula tip of tannic acid were added, the sample was distilled utilizing a 60 cm fractionating column, continuing distillation until 100 mL of distillate was collected. The distillate was cooled in a two-stage process, first passing through a water-cooled condenser followed by passage through a slurry of ice and water before collection in a 100 mL volumetric flask. The distillate was then transferred quantitatively to a 500 mL separating funnel, containing a 35 mm egg-shaped magnet, by repeated rinsing with water. The distillate was then extracted with three portions (10 mL, 5 mL and 5 mL) of dichloromethane by rapid stirring for 10 minutes at a time. The combined dichloromethane fractions were transferred to a pear-shaped vessel with a 1.5 mL graduated stem, to which 0.5 mL of concentrated formic acid was added previously. The dichloromethane extracts were evaporated at room temperature under a stream of nitrogen gas until less than 0.5 mL of concentrate remained. Finally the extract was reconstituted to 1 mL utilizing a solution of 40% acetonitrile in water, homogenized and transferred to a 1.8 mL amber vial for analysis.

5.2.3. Chromatographic details

The LC-MS system utilized was a Waters Alliance 2695 liquid chromatograph (Waters Corporation, Milford, U.S.A.) with Waters Micromass Quattro Premier XE tandem quadrupole mass spectrometric detector (Manchester, U.K.). The concentrated sample extracts were separated by reversed phase liquid chromatography utilizing a methanol and water gradient and a phenyl hexyl separation column (Phenomenex Luna, 250 x 4.6 mm, 3µm) thermostatted at 40°C. The gradient started at 35% methanol, increased to 85% in 18 minutes, followed by a column clean-up step consisting of 95% methanol for 2.5 minutes. The flow was maintained at 1.0 mL/minute throughout the separation and re-equilibration time was 3.5 minutes. Variable injection volumes were used ranging from 5 to 100 µL. Components of interest were introduced into the mass spectrometer utilizing atmospheric pressure chemical ionization (positive ion mode). The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, the experimental parameters are given in Chapter 4 (Table 4.7. and 4.8.).

5.3. Results and discussion

5.3.1. Minimum method criteria

Limited quantitative data available for 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wines indicate levels of IBMP in the range of < 1 to 14 ng/L.[1] Although no corresponding information pertaining to the other major 3-alkyl-2-methoxypyrazines, IPMP and SBMP, are available in the literature, these are expected to be at levels not exceeding approximately 10 to 20% of the total 3-alkyl-2-methoxypyrazines in Sauvignon blanc wine.[2] A comparable study involving a number of Sauvignon blanc wines of Australian, New Zealand and French origin, produced the following results:[2]

Table 5.3.: Methoxypyrazine levels in some Australian, New Zealand and French Sauvignon blanc wines.[2]

Methoxypyrazine	Levels (ng/L)
IPMP	0.9 to 5.6
IBMP	0.6 to 38.1
SBMP	0.1 to 1.0

It should be noted that the limit of detection of the analytical method used in this study was 0.15 ng/L and that IPMP and SBMP were not detected in some of the wines. The methoxypyrazine levels in New Zealand wines were also significantly (and consistently) higher than in Australian wines, a phenomenon ascribed to the fact that fruit grown under cool conditions generally produces higher methoxypyrazine levels.[2,3,4]

In order to characterize the profile of abundance of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc, the analytical method should at least be capable of accurate determination of the three important methoxypyrazines namely IPMP, IBMP and SBMP. Method detection levels in the order of 0.03 ng/L and minimum quantification levels in the order of 0.10 ng/L may be considered minimum requirements in terms of sensitivity for the purpose of this study. The method accuracy, expressed as recovery, should be better than 80%. As a single 750 mL bottle is available per sample and 500 mL of wine is consumed in the sample preparation procedure, the precision of the method must be such that reliable results may be produced from a single determination per sample. The intra-assay precision, expressed as the RSD of the recovery, should therefore be better than 10%.

5.3.2. Determination of the linear working range

A linearity study utilizing standards was performed to demonstrate linear system response over the range of expected residue levels. Quantities ranging from extinction levels to 4000 pg on column were injected, which corresponded to a maximum sample residue level of 80 ng/L, a concentration factor of 500 and a 100 µL injection. The mass of analyte injected was plotted against measured peak area as well as response factor (peak area/mass). The results of the linearity study are summarized in Tables 5.4. to 5.6.

Table 5.4.: Linearity, IPMP primary and secondary ion transitions (152.9 to 137.9 and 122.9 respectively).

Mass (pg)	Average peak area /% RSD (n = 3, 152.9 > 137.9)	Response factor (area / mass)	Average peak area /% RSD (n = 3, 152.9 > 122.9)	Response factor (area / mass)
0.6150	15 / 74%	24	32 / 46%	52
1.230	47 / 58%	38	63 / 14%	51
2.460	55 / 55%	22	81 / 43%	33
6.150	161 / 16%	26	150 / 26%	24
24.60	873 / 9%	35	768 / 12%	31
123.0	3937 / 4%	32	3545 / 1%	29
369.0	11940 / 4%	32	10446 / 3%	28
1230	40482 / 5%	33	36899 / 6%	30

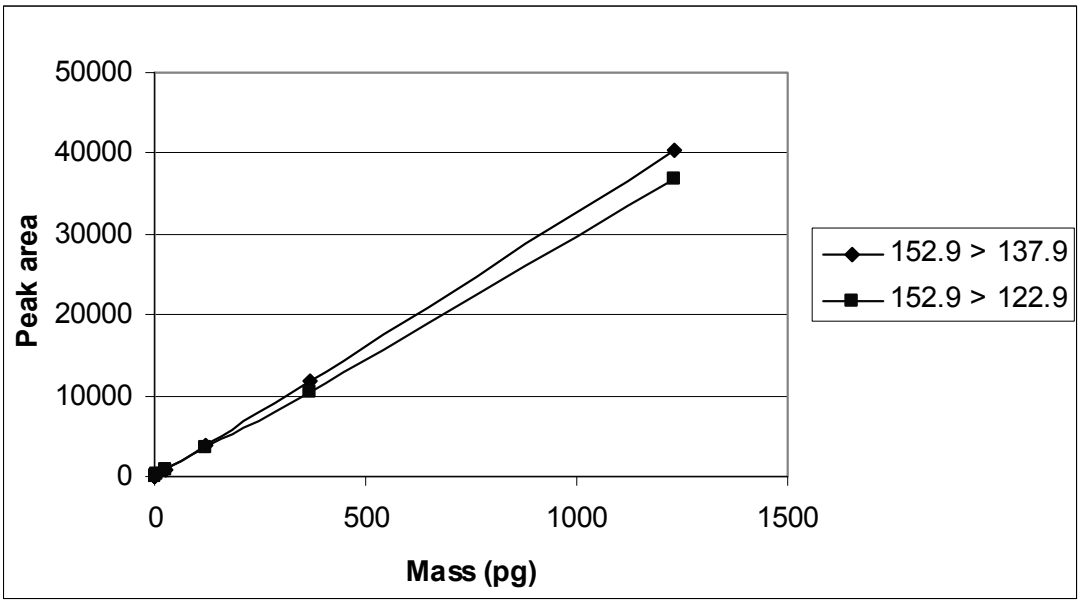

Figure 5.1.: Linearity, IPMP (mass versus peak area) for primary as well as secondary ion transitions.

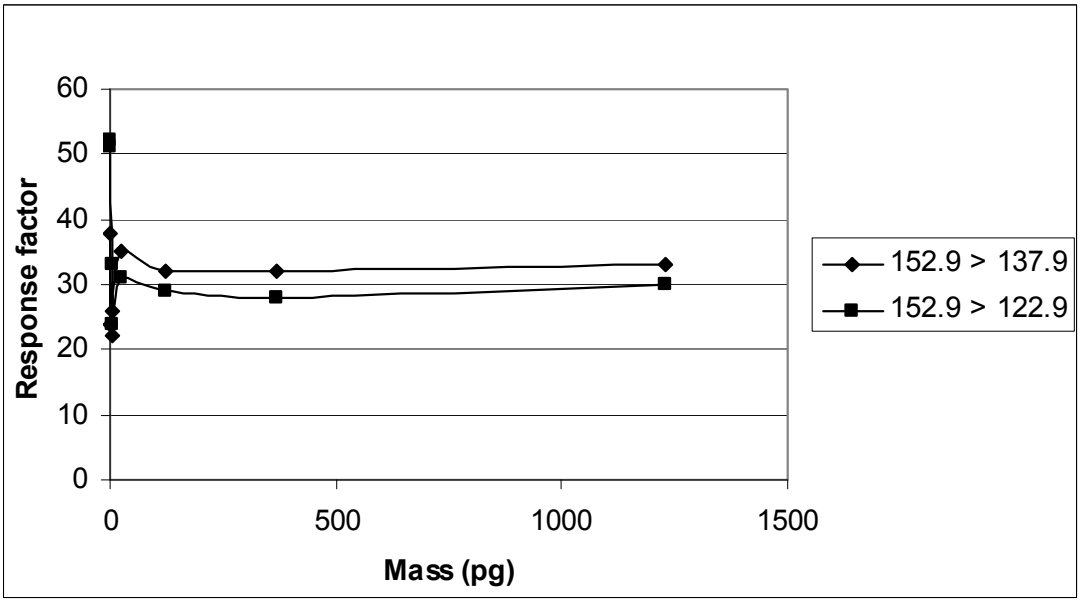

Figure 5.2.: Linearity, IPMP (mass versus response factor).

Table 5.5.: Linearity, IBMP primary and secondary ion transitions (167.0 to 125.0 and 124.0 respectively).

Mass (pg)	Average peak area /% RSD (n = 3, 167.0 > 125.0)	Response factor (area / mass)	Average peak area /% RSD (n = 3, 167.0 > 124.0)	Response factor (area / mass)
2.384	110 / 32%	46	70 / 29%	29
4.768	280 / 15%	59	186 / 19%	39
9.536	582 / 17%	61	405 / 7%	42
23.84	1456 / 8%	61	1152 / 5%	48
95.36	6434 / 3%	67	4753 / 4%	50
476.8	29483 / 1%	62	21028 / 1%	44
1430	93214 / 1%	65	67438 / 1%	47
4768	306207 / 4%	64	224342 / 4%	47

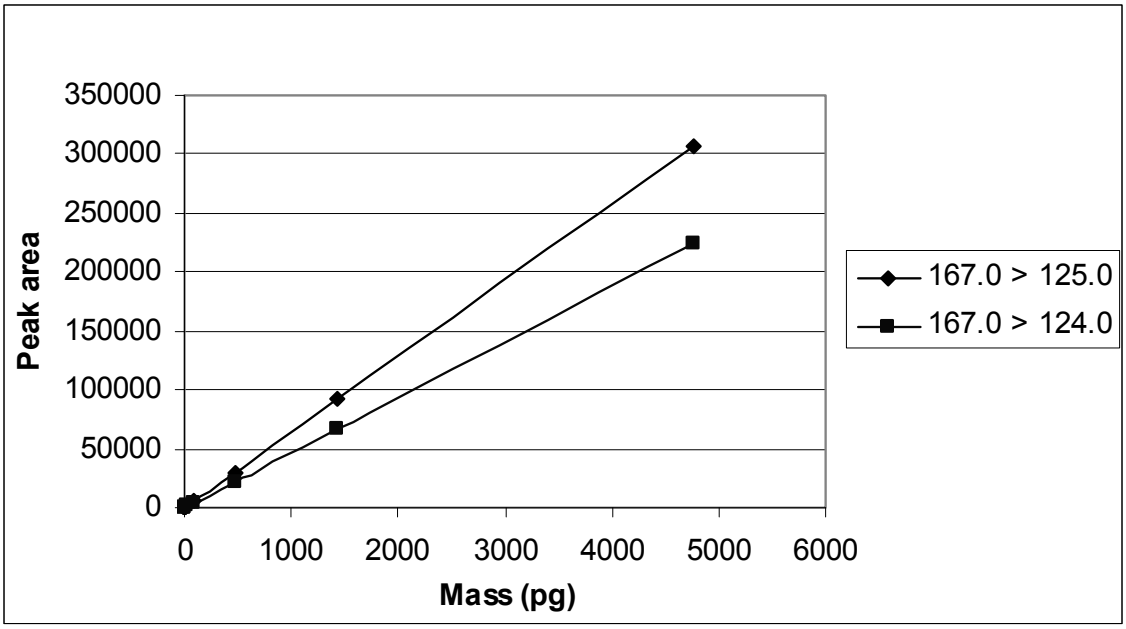

Figure 5.3.: Linearity, IBMP (mass versus peak area).

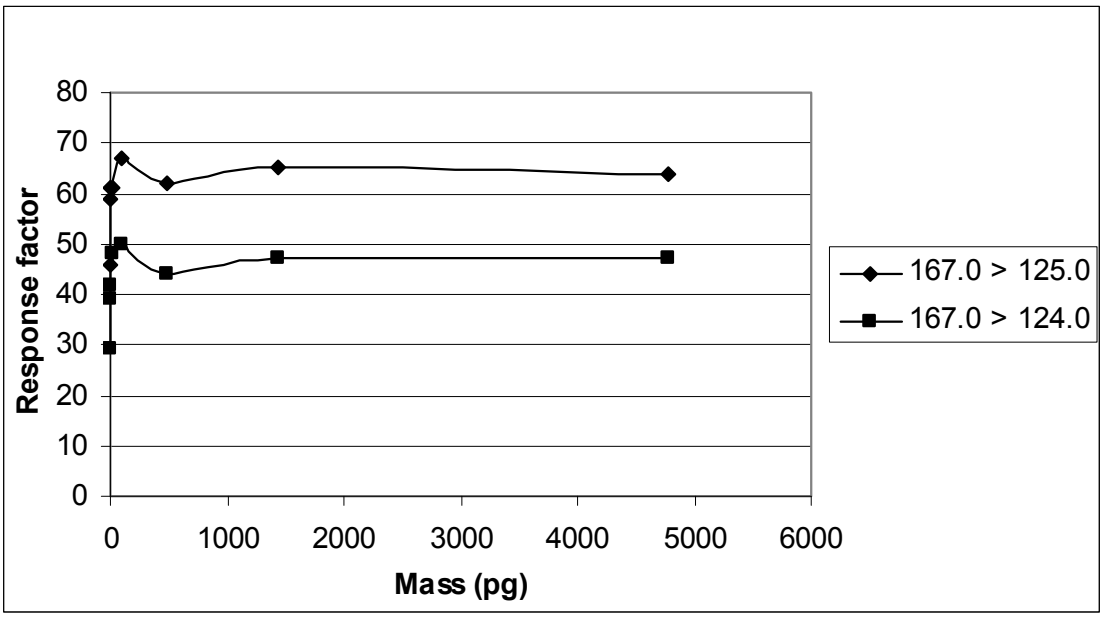

Figure 5.4.: Linearity, IBMP (mass versus response factor).

Table 5.6.: Linearity, SBMP primary and secondary ion transitions (167.0 to 138.0 and 123.0 respectively).

Mass (pg)	Average peak area /% RSD (n = 3, 167.0 > 138.0)	Response factor (area / mass)	Average peak area /% RSD (n = 3, 167.0 > 123.0)	Response factor (area / mass)
0.604	22 / 72%	36	38 / 80%	63
1.208	87 / 34%	72	38 / 37%	31
2.416	182 / 12%	75	79 / 51%	33
6.040	409 / 3.6%	68	216 / 22%	36
24.16	1896 / 3%	78	985 / 9%	41
120.8	8469 / 2%	70	4566 / 6%	38
362.4	26541 / 3%	73	14554 / 2%	40
1208	84078 / 3%	70	46052 / 4%	38

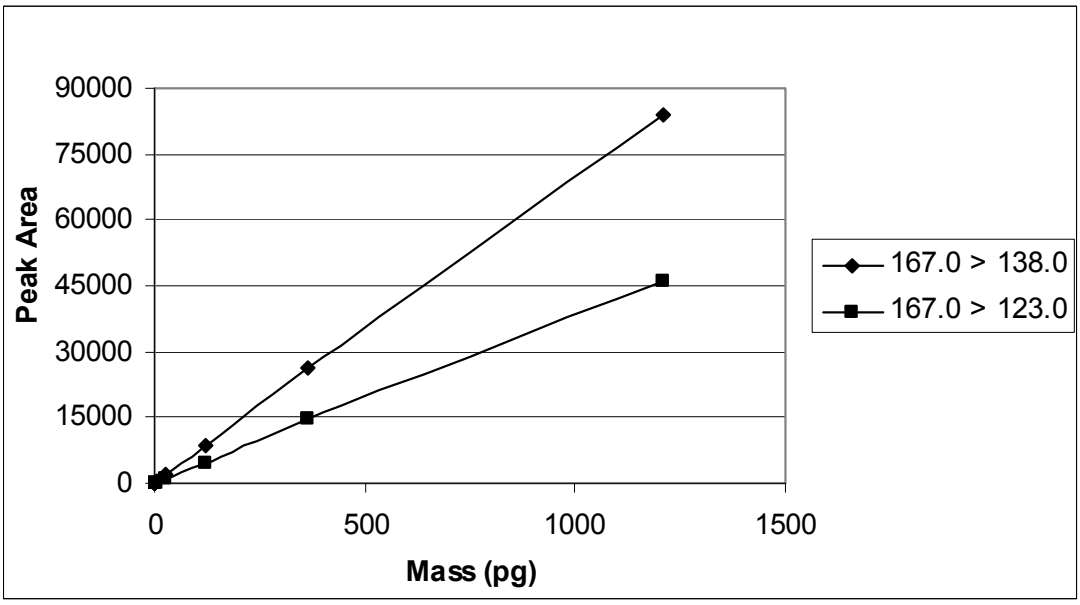

Figure 5.5.: Linearity, SBMP (mass versus peak area).

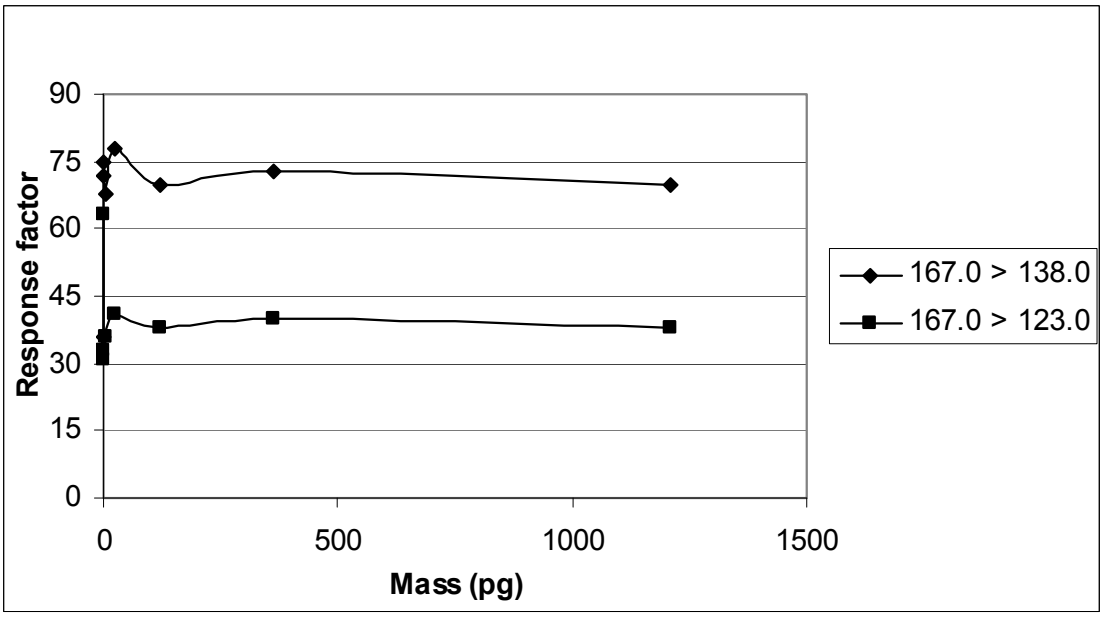

Figure 5.6.: Linearity, SBMP (mass versus response factor).

The Pearson's correlation coefficients[5], r, were determined from the limit of quantification (5 pg) to highest injected masses in each case and were as follows Table 5.7.:

Table 5.7.: Pearson's correlation coefficients for the calibration curves of the compounds of interest.

Compound	IPMP	IBMP	SBMP
Primary transition	0.99998	0.99998	0.99987
Secondary transition	0.99985	0.99998	0.99987

The system response was found to be linear between the quantification limit and 4 000 pg and 1000 pg on-column for IBMP, and IPMP and SBMP respectively. Quantification of the said compounds should therefore strictly be performed within this mass range. When actual samples are analyzed, the system may be calibrated over a smaller concentration range which is better suited to the determination of the substances under investigation at realistic sample concentrations. Where the sample concentration exceeds the established working range, appropriate injection volumes should be used to ensure that the mass injected falls within the actual calibration range.

5.3.3. Limits of detection and quantitation

The limits of detection (MDL) and of quantitation (MQL) of the method for the substances under investigation were obtained from the linearity study. These values were estimated based on the smallest amount of solute that produced a signal equivalent to three times and ten times of the average noise, respectively. The data processing software (Masslynx 4.0) was used to calculate the signal-to-noise ratios using unsmoothed chromatograms for each of the MRM signals. At the minimum quantifiable level, the ratios of all ions used for identification and quantitation were found to be consistent with those produced at higher concentrations (Table 5.9.). Measured minimum detectable quantities obtained correspond to sample residue levels of 0.03 ng/L, while the minimum quantification limits correspond to levels of 0.10 ng/L, considering sample pre-concentration of a factor of 500 and injection volumes of 100 µL.

Table 5.8.: Minimum detectable and quantifiable levels obtained with the method.

Methoxypyrazine	MDL (pg on column)	MQL (pg on column)
IPMP	1.5	5.0
IBMP	1.5	5.0
SBMP	1.5	5.0

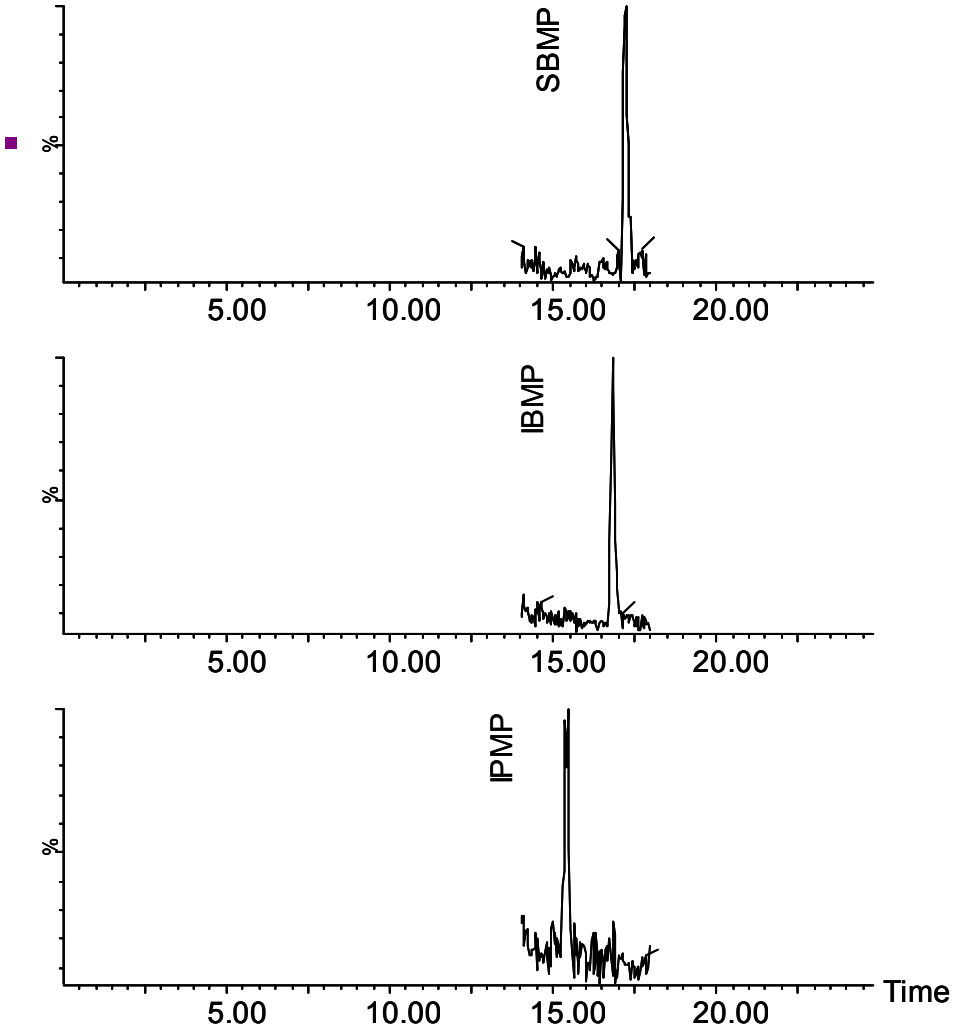

Figure 5.7.: Chromatogram of a standard solution containing the three 3-alkyl-2-methoxypyrazines under investigation close to the limit of quantitation. IPMP = 6.2 pg, IBMP = 23.8 pg, SBMP = 6.0 pg.

5.3.4. Method specificity

The three target methoxypyrazines were identified utilizing retention times as well as the ratios of abundance measured for the relevant primary- and secondary ions. Table 5.9. presents the relevant data used for peak identification (obtained from calibration standards).

Table 5.9.: Criteria for peak identification.

Methoxypyrazine	Retention time (minutes)	Primary transition	Secondary transition	Ratio of ions
IPMP	15.39	152.9 > 137.9	152.9 > 122.9	1.11
IBMP	16.81	167.0 > 125.0	167.0 > 124.0	1.37
SBMP	17.21	167.0 > 138.0	167.0 > 123.0	1.88

Some European Union guidelines for compound identification by MS/MS require that a precursor parent ion together with two daughter ions (and one ion ratio) be obtained for confirmatory purposes. The relative daughter ion intensities, expressed as a percentage of the intensity of the most intense ion, must be consistent with that of the reference standards (at comparable concentrations). The relevant retention times obtained for the analytes in the sample should also match those of the reference standards. The maximum allowable tolerance regarding the ion ratios is approximately ± 20% while retention times should correlate within 2.5%.[6]

The compounds of interest, determined in wine samples (unfortified wine as well as fortified to 1, 10 and 100 ng/L) as part of the recovery study, produced retention time and ion ratio values (Table 5.10.) that were consistent with the identification criteria outlined above. These criteria (Table 5.9.) were therefore also applied for compound identification in this study.

Table 5.10.: Peak identification parameters obtained for fortified wine samples in the recovery study (n = 36).

Methoxypyrazine	Retention time (minutes)	RSD (%) of retention time	Ratio of ions	RSD (%) of ion ratios
IPMP	15.35	0.25	1.13	6.5
IBMP	16.78	0.17	1.39	3.2
SBMP	17.17	0.20	1.92	8.1

5.3.5. Method accuracy

The accuracy of the method was assessed by determination of the recovery of the substances under investigation from samples of Sauvignon blanc wine, suitably fortified prior to the sample preparation step. Nine replicate sets of recovery measurements were made at three levels of fortification, 1 ng/L, 10 ng/L and 100 ng/L. The results of the recovery study, including overall recovery over the range of levels investigated, are presented in Table 5.11.

Table 5.11.: Recovery study at three levels of fortification (1, 10 and 100 ng/L) as well as overall recovery (average recovery over all levels investigated).

1 ng/L, n = 9	IPMP	IBMP	SBMP
average (%)	95.1	100.7	93.5
RSD (%)	7.1	10.3	5.8
10 ng/L, n = 9	IPMP	IBMP	SBMP
average (%)	90.8	97.3	94.4
RSD (%)	11.6	6.0	5.7
100 ng/L, n = 9	IPMP	IBMP	SBMP
average (%)	89.3	96.1	93.6
RSD (%)	6.5	4.7	4.3
OVERALL, n = 27	IPMP	IBMP	SBMP
average (%)	91.8	98.0	93.8
RSD (%)	8.8	7.5	5.1

5.3.6. Method precision

The reproducibility of the LC-MS instrument was determined by performing repeated injections of a standard solution containing the three methoxypyrazines. Reproducibility was expressed as the standard deviation of the peak area obtained for the components of interest (Table 5.12.).

Table 5.12.: Instrument precision obtained for the LC-MS system upon injection of a standard (n = 7).

	IPMP (61.5 pg)	IBMP (238.4 pg)	SBMP (60.4 pg)
average	2181	15699	4404
RSD (%)	2.9	1.5	4.3

The intra-assay precision was assessed by considering the repeatability obtained in the recovery study. The recovery of the substances under investigation was better than 90% with RSD of less that 10% at concentrations of 1 ng/L, 10 ng/L and 100 ng/L.

5.3.7. Uncertainty of measurements and reporting of results

The uncertainty of measurements associated with the procedure was estimated by calculating the range of the 95% confidence interval (C.I.) about the measurements performed in the recovery study (i.e. at levels of 1 ng/L, 10 ng/L and 100 ng/L). The confidence limits (C.L.) pertaining to the recovery of the target species were calculated using the following equation:[7]

$$95\%\ \text{C.L.} = \text{mean value} \pm zs / (n)^{1/2}$$

Where z (95% C.L.) is 1.96, s is the standard deviation, and n the degrees of freedom. The range of the 95% C.I. about the recovery measurements made at the average of three levels of fortification was as follows:

Table 5.13.: Calculation of 95% confidence limits.

Concentration level 1 to 100 ng/L	n	s (%)	z (95% C.L.)	Mean (%)	C.I. (%)
IPMP	(27 – 1)	8.0	1.96	91.8	3.1
IBMP	(27 – 1)	7.3	1.96	98.0	2.8
SBMP	(27 – 1)	4.8	1.96	93.8	1.9

Results were reported using the significant figure convention, according to which the result is reported by recording all the certain digits and the first uncertain digit.[5,7,8] As the limit of quantification of the procedure was determined to be a tenth of 1 ng/L, and the recoveries of the order 95 ± 3%, all results were reported in units of ng/L and by recording two significant figures.

5.3.8. System stability

The long-term stability of the system was evaluated upon completion of the investigation by observing the response obtained for the same standard in the routine calibration of the system prior to sample analysis. System stability was demonstrated by plotting the peak area obtained for 238.4 pg IBMP as a function of calibration date. It is evident from the information in Figure 5.8. that the system response was constant over the entire period of the investigation. The variation, expressed as % RSD of the peak area, was 6.9% over the three-month period (August to October) that the samples were analyzed. During this period the system was calibrated 16 times and approximately 700 wine samples were analyzed.

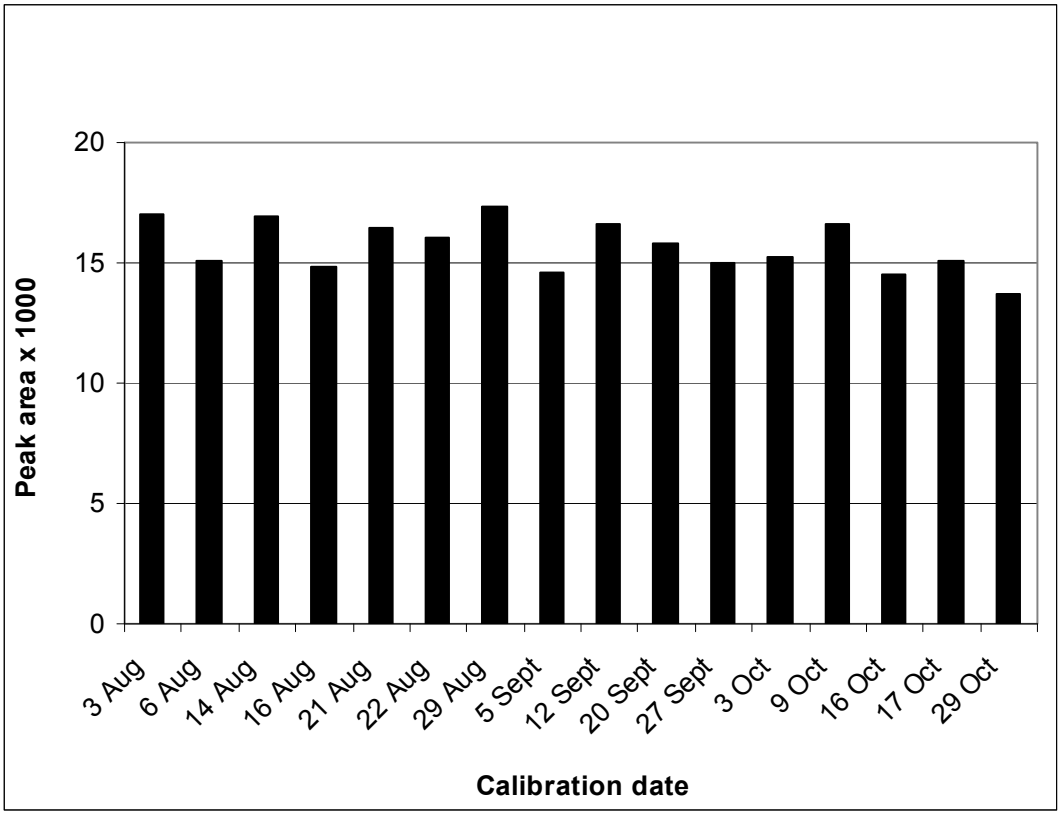

Figure 5.8.: System stability over the period of the investigation (August to October) expressed as the peak area of 238.4 pg IBMP on column.

5.4. Conclusions

The validation study revealed that the method met the minimum requirements as outlined in section 5.3.1. and that it is therefore suitable for the routine trace level determination of the said compounds in Sauvignon blanc wine.

The recoveries obtained indicate that the sample preparation procedure, described in Chapter 3, produces precise (RSD less than 10%) pre-concentration of the analytes with good recovery (better than 90%). The precision of the LC-APCI-MS method was equally good (RSD less than 5%) while linear system

response was demonstrated from 5 to 4000 pg injected. The MDL and MQL for the method were determined to be 0.03 and 0.10 ng/L respectively.

The performance characteristics of this LC-MS method were generally better than that of all published GC methods utilized for the same purpose. The GC methods produced MDL values ranging from 0.15 to 2 ng/L while MQL values in the order of 2 ng/L were reported. These latter techniques are therefore at least five times less sensitive than the described LC-MS method.

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CHAPTER 6

The contents of some 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine and detection of adulteration

6.1. Introduction and objectives

The overall objectives of this study were in the first instance to develop and implement a method for the determination of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine at their natural levels of occurrence and secondly to analyze a spectrum of wines, representative of the South African Sauvignon blanc industry. A comprehensive database of the 3-alkyl-2-methoxypyrazine content of South African Sauvignon blanc wine may thus be established, and used to implement a protocol for unequivocal identification of adulterated wines regarding addition of foreign sources of 3-alkyl-2-methoxypyrazines.

Residues of the three major 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wines were analyzed by liquid chromatography with mass spectrometric detection. The method yielded qualitative (confirmatory) information pertaining to the components of interest in addition to quantitative information. Three 3-alkyl-2-methoxypyrazenes, 3-isobutyl-2-methoxypyrazine (IBMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP) and 3-isopropyl-2-methoxypyrazine (IPMP) were thus investigated and quantified. The method yielded limits of detection and quantification of 0.03 ng/L and 0.10 ng/L, respectively.

Two parameters were identified that may be used to distinguish adulterated wines. The IBMP concentration, despite showing considerable variance, was

confined to a range spanning approximately two orders of magnitude (0.40 to 44 ng/L). Possible maximum values for this compound in South African Sauvignon blanc wines may therefore be established and classification of the wine according to factors such as the region of origin and vintage attempted. Furthermore, it was found that South African Sauvignon blanc wines contain the three analysed pyrazenes in distinct relative amounts and that these ratios may therefore be used to elucidate authenticity. In addition, the effect of adulteration with green pepper extracts or synthetic flavor preparations on the relative abundances were evaluated by characterizing the corresponding profiles in green pepper extracts and synthetic flavor preparations. The analytes were also determined in Sauvignon blanc vine leaves and in a small number of wines of other cultivars.

The data was analyzed utilizing multivariate and univariate statistical methods to investigate possible grouping of samples in the dataset and to distinguish adulterated wines. Principal component analysis (Statistica version 7 and 8) and Anova (Statistica version 8) were used to perform data analysis. The results for Anova are discussed concurrently with that of PCA.

6.2. Materials and methods

6.2.1. Samples

A total of 577 South African Sauvignon blanc wines, from all wine-producing regions and of vintages 1999 to 2007, were analyzed. A number of wines produced form other grape cultivars (roughly 6 per cultivar, recent vintages) were also analyzed. Samples were obtained from submissions under the South African controlled appellations system (South African Wine and Spirit Board) as well as from export applications (South African National Department of Agriculture). Two samples suspected to be adulterated with green pepper extracts or synthetic preparations, were also confiscated earlier by the Department of Agriculture

under the provisions of the Liquor Products Act.[1,2] In order to maintain confidentiality, information pertaining to individual producers and estates are omitted.

Green peppers were obtained from the local trade and were not purchased from the same vendor or on the same date. The synthetic flavor preparations were from The Duckworth Group (Killarney Gardens, South Africa) while Sauvignon blanc vine leaves were obtained from the IWBT, Stellenbosch University.

Additional information pertaining to the chemical analysis of the Sauvignon blanc wine samples were obtained from archived data that were used for certification of the products under the South African controlled appellations system. Methods of analysis used to obtain this data were as follows: Alcohol content by densiometry, total reducing sugars by flow-injection analysis, total acidity by titration and the pH was measured with a pH meter.

6.2.2. Sample preparation

Aliquots of wine samples (500 mL) were distilled (60 cm fractionating column) followed by exhaustive liquid extraction with dichloromethane. The combined dichloromethane fractions (stabilized with formic acid) were then evaporated and reconstituted to 1 mL with a solution of 40% acetonitrile in water and transferred to a 1.8 mL amber vial for analysis. A detailed description of the sample preparation process is given in Chapter 3.

The extraction of 3-alkyl-2-methoxypyrazines from vegetable materials (green peppers and leaves) was based on the validated procedure that was used for wine. The material (approximately 25 g) was weighed on an analytical balance and manually ground to a pulp utilizing a pestle and mortar. The pulp was then transferred to a boiling flask by rinsing with a solution of 10% ethanol. To the

contents of the flask was added 100 mL of a solution of 10% ethanol, a spatula tip of tannic acid and a boiling stone, followed by distillation of the solution. The distillate thus obtained (50 mL) was extracted and concentrated similarly to the wine distillates.

The synthetic preparations were analyzed after weighing followed by serial dilution to yield an appropriately diluted sample.

6.2.3. Chromatographic details

The analyses were carried out using a Waters Alliance 2695 liquid chromatograph (Waters Corporation, Milford, U.S.A.) with Waters Micromass Quattro Premier XE tandem quadrupole mass spectrometric detector (Manchester, U.K.). The samples were separated by reversed phase liquid chromatography utilizing a methanol and water gradient and a phenyl hexyl separation column (Phenomenex Luna, 250 x 4.6 mm, 3 µm) thermostatted at 40°C. The gradient started at 35% methanol and increased to 85% in 18 minutes, followed by column clean-up and re-equilibration. The flow was 1.0 mL/minute and the total run-time 25 minutes. Components of interest were ionized utilizing atmospheric pressure chemical ionization in the positive ion mode. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, acquiring data for two ion transitions as described in Table 6.1. Ion ratios thus obtained served to positively identify the substances under investigation. Complete LC-MS method details are given in Chapter 4.

Table 6.1.: MRM transitions used in the LC-APCI-MS method for determination of some 3-alkyl-2-methoxypyrazines.

Compound	Parent Ion (Da)	Daughter Ion (Da)	Transition	Cone (V)	Collision (eV)
IPMP	152.9	122.9	2 °	34	26
	152.9	137.9	1 °	34	18
IBMP	167.0	124.0	2 °	34	22
	167.0	125.0	1 °	34	16
SBMP	167.0	123.0	2 °	34	24
	167.0	138.0	1 °	34	18

6.2.4. Statistical methods

The complete dataset pertaining to Sauvignon blanc wine analyzed for 3-alkyl-2-methoxypyrazines, generated by this investigation, consisted of 577 samples. In a number of cases the IPMP and SBMP content were below the MQL (0.10 ng/L) of the method and these samples were removed from the dataset prior to statistical analysis of the data. Statistica (Statsoft Inc., OK, US, versions 7 and 8) was used to perform statistical analyses. Analysis of variance (Anova) was performed following 'bootstrap' correction to correct for non-gaussian distribution of the data. Quantitative data were standardized to produce variables with 0 mean and 1 standard deviation prior to principal component analysis (PCA).

6.3. Results and discussion

6.3.1. Sauvignon blanc wines

6.3.1.1. Classification of analysed wines

Of the 577 Sauvignon blanc wines analysed in the current study, the majority (82%) of the samples were of the 2004 and 2005 vintages. Due to the

unavailability of samples, other vintages such as 1999 to 2002 and 2007 were less well represented (only one sample each for vintages 1999 to 2001 were analysed). Sample distribution over these vintages are summarised in Figure 6.1.

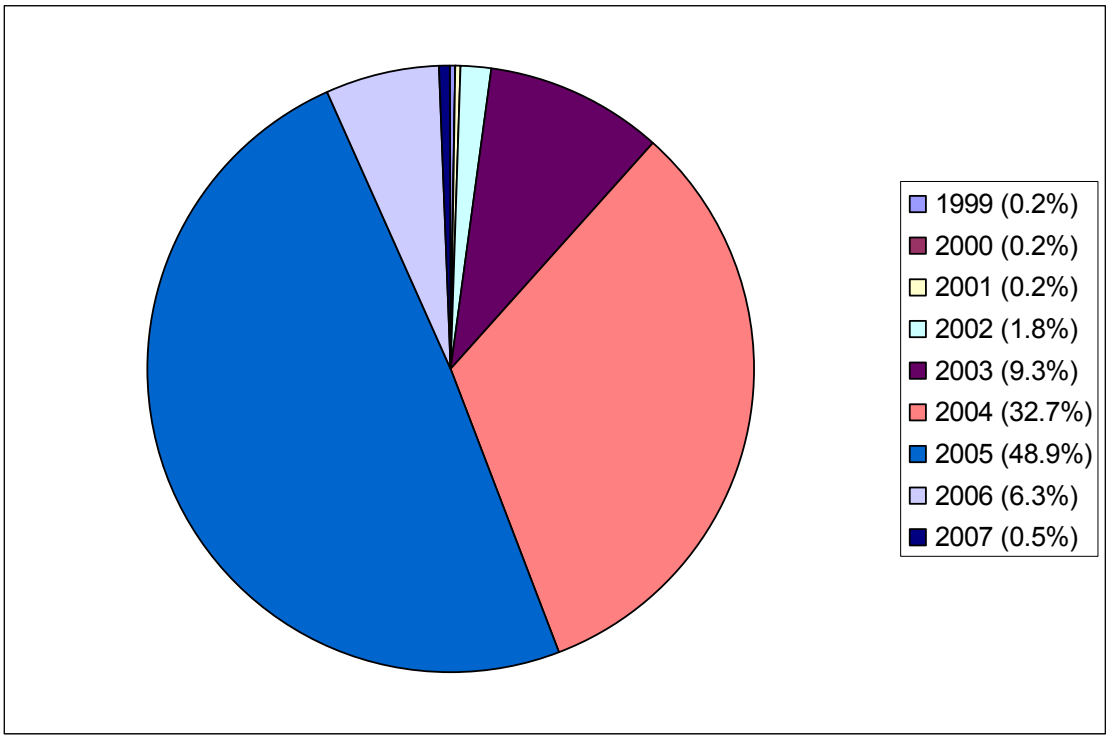

Figure 6.1.: Vintage distribution of the analyzed Sauvignon blanc wine samples (percentage representation in parenthesis).

The South African Wine of Origin Scheme guarantees the validity of the geographical delimitation on the labels of all certified products. Under this scheme, the wine-producing areas of South Africa are divided into a number of regions, districts and wards.[1,3] Figure 6.2. presents a map of the most important wine producing regions of South Africa. The majority of South African Sauvignon blanc vineyards are found in the Coastal and Breede River valley regions.[4] The bulk of the samples in this investigation were also from these regions. A list of represented areas as obtained from bottle labels is presented in Table 6.2. and in Table 6.3. the wine samples are grouped according to the latest regional/district classification.

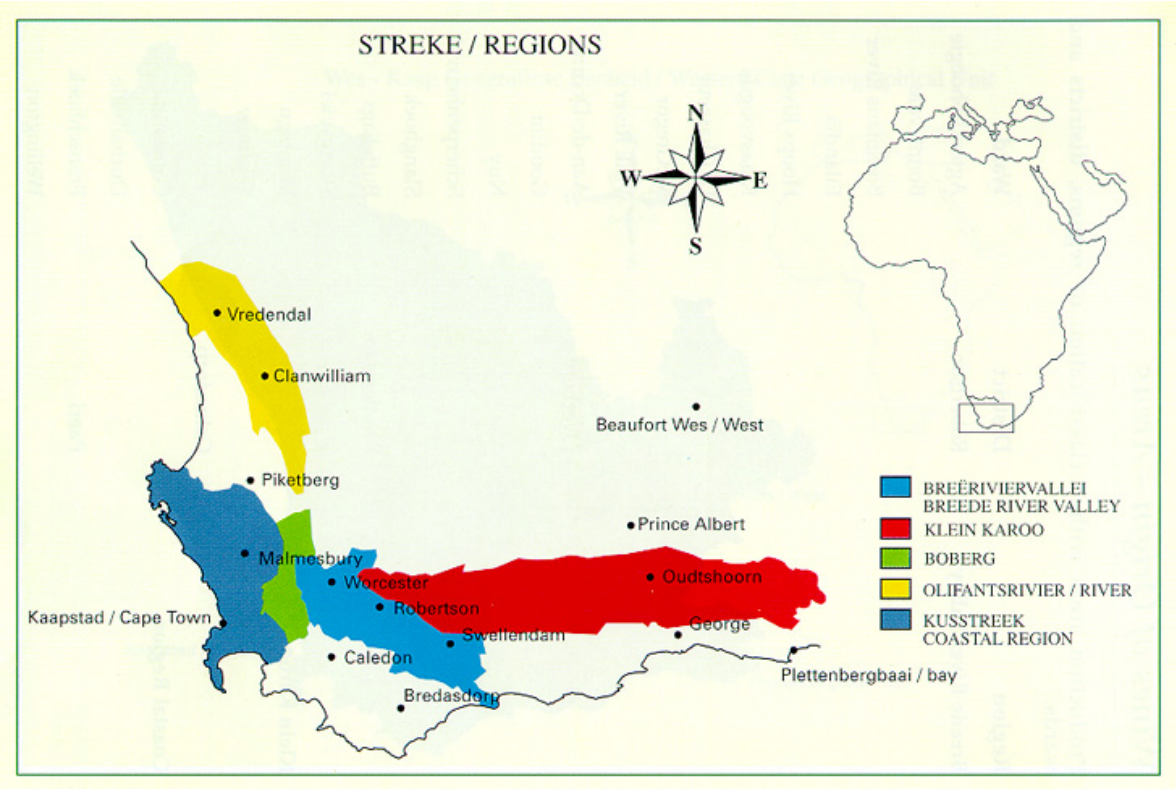

Figure 6.2.: Wine producing regions of South Africa.

Table 6.2.: Distribution of samples over the wine-producing regions of South Africa according to label classification.[1]

No.	Classification on label.	No. of samples.
1	Bonnievale	1
2	Bottelary	4
3	Breede River Valley	5
4	Cape Agulhas	5
5	Cape Point	2
6	Cederberg	2
7	Constantia	16
8	Darling	5
9	Devon Valley	1
10	Durbanville	20
11	Elgin	9
12	Elim	7
13	Franschhoek	12
14	Goudini	12
15	Groenekloof	5
16	Klein Rivier	1
17	Coastal Region	65
18	Lutzville	2
19	McGregor	3
20	Nuy	5
21	Paarl	25
22	Prince Albert Valley	1
23	Robertson	54
24	Simonsberg	12
25	Slanghoek	5
26	Stellenbosch	88
27	Swartland	3
28	Tradouw	1
29	Tulbagh	2
30	Walker Bay	9
31	Wellington	8
32	Western Cape**	153
33	Worcester	33

** = includes all wine producing regions of the Western Cape.

Table 6.3.: Distribution of samples according to the latest regional/district classification.[3]

No.	Region/district.	No. of samples.
1	**Breede River Valley**	**118**
2	- Breedekloof	17
3	- Robertson	58
4	- Worcester	38
5	**Coastal Region**	**268**
6	- Cape Point	2
7	- Constantia	16
8	- Darling	10
9	- Paarl	45
10	- Tygerberg	20
11	- Stellenbosch	105
12	- Swartland	3
13	- Tulbagh	2
14	**Little Karoo**	**1**
15	- Tradouw	1
16	**Olifants River**	**2**
17	- Lutzville Valley	2
19	Cape Agulhas	12
20	Overberg	10
21	Walker Bay	9
22	Cederberg*	2
23	Prince Albert Valley*	1
24	Western Cape**	153

* = wards

** = includes all wine producing regions of the Western Cape.

From the information in Table 6.3. it is evident that of the samples that originated from a specific area, approximately 91% were from the Breede River Valley and Coastal regions, where most Sauvignon blanc vineyards are located. Sampling may therefore be considered as reasonably representative of the overall South African Sauvignon blanc production as far as geographical origin is concerned.

6.3.1.2. Interpretation of quantitative 3-alkyl-2-methoxypyrazine data: Elucidation of criteria for authenticity

Quantitative data for the three major 3-alkyl-2-methoxypyrazines obtained in the study are presented in Appendix 2 of this thesis. From these data it was concluded that IBMP is the major 3-alkyl-2-methoxypyrazine in South African Sauvignon blanc wine. This observation is in agreement with the finding of various other reports on this subject.[5,6,7] It has also been reported that IBMP is the principal contributor to the vegetal aroma in Sauvignon blanc wine.[6] As IBMP is also the most abundant 3-alkyl-2-methoxypyrazine in green peppers[8], it is apparent that the levels of this compound may be attenuated by the addition of green pepper extracts to Sauvignon blanc wine.

The concentration of IBMP was therefore identified as a potential parameter that may be utilized to detect adulteration of South African Sauvignon blanc wine. In this study, measured values for the IBMP concentration in 575 South African Sauvignon blanc wines ranged from 0.40 to 44 ng/L with an average of 6.2 ng/L. Lacey *et al.* [7] reported values of 0.6 to 38 ng/L in a study of 22 Sauvignon blanc wines of Australian, New Zealand and French origin. New Zealand Sauvignon blanc wine contained consistently higher levels of IBMP with an average of 26 ng/L compared to 6.8 ng/L for Australian wines. A study conducted by Allen *et al.*[6] on eight commercial Sauvignon blanc wines of Australian and New Zealand origin produced values of 4.7 to 33 ng/L for the IBMP concentration.

It has tentatively been reported that Sauvignon blanc wine as well as some red wines contain the three major 3-alkyl-2-methoxypyrazines in fairly constant relative amounts.[7,9] Lacey *et al.* [7] reported that Sauvignon blanc wines in particular display a ratio of 7:1 (SD = 1.9) for IBMP to IPMP. As IBMP has been reported to be far more dominant relative to the other 3-alkyl-2-methoxypyrazines in green peppers (approximately 100:1)[8], it follows that adulteration of wine with this commodity may enrich the levels of IBMP disproportionately. The ratios of

relative abundance of the three major 3-alkyl-2-methoxypyrazines may therefore reveal this form of adulteration. In this study it was determined that the levels of IPMP varied between < 0.03 and 3.9 ng/L while that of SBMP varied between < 0.03 and 3.2 ng/L. In the study conducted by Lacey *et al.* [7] concentrations of IPMP ranging between 0.9 and 5.6 ng/L and of SBMP ranging between 0.1 and 1.3 ng/L were reported. Allen *et al.* [6] reported IPMP concentrations up to 3.8 ng/L. It should be noted that neither of these authors were able determine IPMP or SBMP in all the wines that were investigated and that these values therefore pertain to a subset of the wines in these respective studies.

The improved level of sensitivity (LOQ = 0.10 ng/L) attained with the analytical method developed in this study facilitates the quantitative determination of three major 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine. The method is therefore suited to produce data that permits the implementation of both strategies outlined above.

6.3.1.3. Determination of limits for the occurrence of IBMP in South African Sauvignon blanc wines

The concentrations of IBMP in South African Sauvignon blanc wines are influenced by a multitude of factors. A close relationship between the climate of the area and the season and the levels of IBMP in South African Sauvignon blanc wine has been reported. This grape variety is also sensitive to viticultural and production factors.[5,10,11] This has been attributed to the temperature and light sensitivity of the 3-alkyl-2-methoxypyrazines.[5,10] Significant variance may therefore be expected for the concentrations of IBMP in South African Sauvignon blanc wines. Minimum and maximum values found in this investigation were 0.40 and 44 ng/L respectively while the average of all samples was 6.2 ng/L with RSD of 102% (n = 575). The median was determined to be 4.3 ng/L, indicating that the

majority of samples are at the lower end of the scale. The distribution of IBMP levels in the analyzed wines is presented in Figure 6.3.

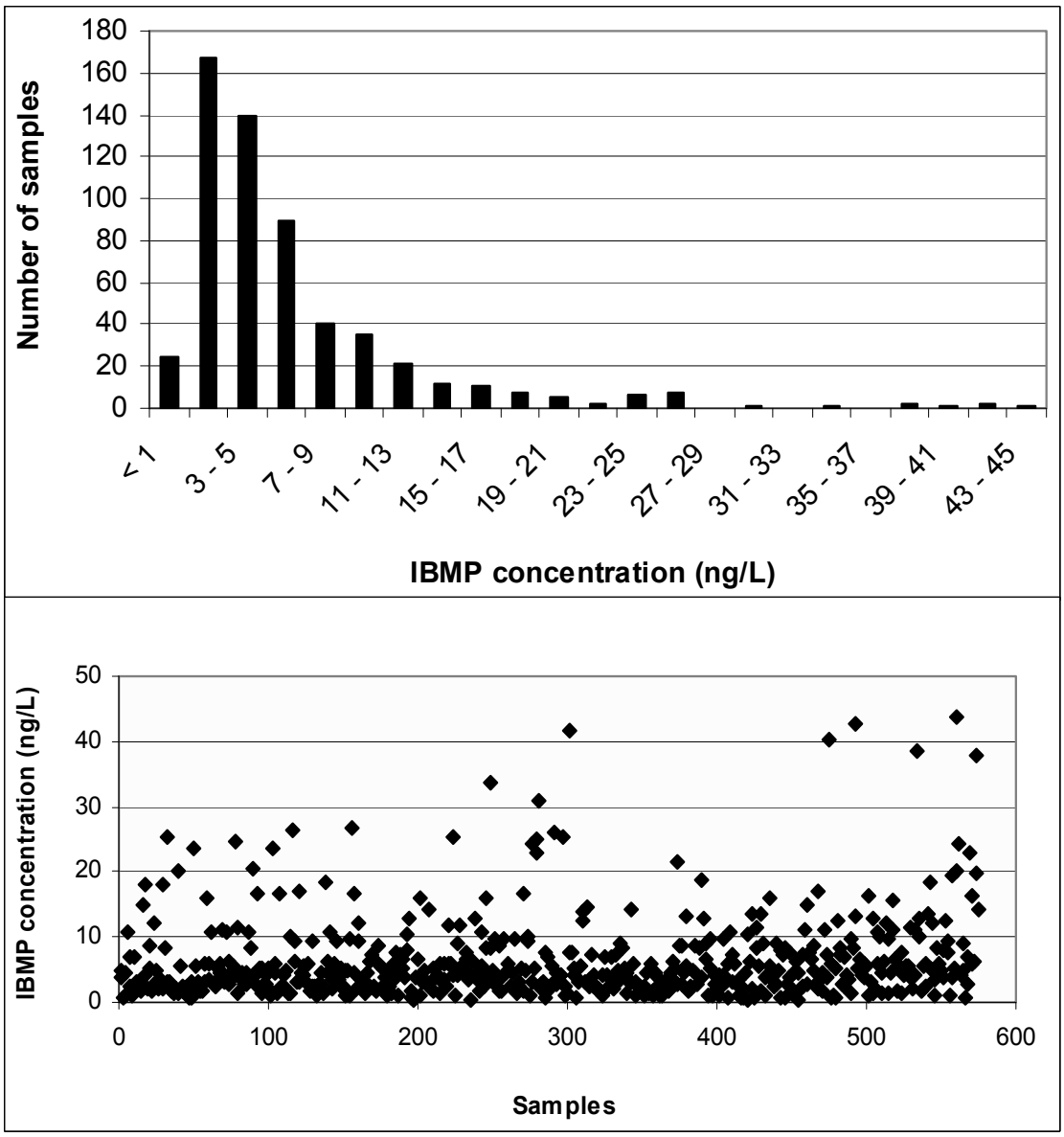

Figure 6.3.: Distribution of IBMP levels in 575 analyzed South African Sauvignon blanc wines: Number of samples as a function of concentration (top), and (bottom) concentration of IBMP in each of the samples (Appendix 2).

These results show a relatively large variation (RSD = 102%) in the IBMP concentration across all analyzed wines. It is also evident that the highest number of samples contains between 1 and 3 ng/L IBMP and that the frequency decreases from that up to the maximum concentration of 44 ng/L. It is therefore clear that the regions and vintages should be considered separately in order to attempt a more accurate classification of the limits for this compound, taking into consideration the effect of each of these parameters. Table 6.4. presents a summary of the IBMP content of vintages 2002 to 2006 for wines grouped according to geographical origin while Table 6.5. presents the same information for each vintage.

Table 6.4.: IBMP levels and standard deviation (ng/L) in Sauvignon blanc wines from different wine-making regions over vintages 2002-2006 (minimum / maximum / average / number of samples).

Region/district	All vintages	Standard deviation
Breede River Valley	0.66 / 26 / 5.8 / 118	6.0
- Breedekloof	2.6 / 26 / 8.6 / 17	6.8
- Robertson	0.66 / 26 / 5.8 / 58	7.2
- Worcester	1.3 / 17 / 4.6 / 38	3.6
Coastal Region	0.40 / 38 / 6.4 / 267	5.0
- Constantia	6.0 / 20 / 11 / 16	4.2
- Darling	4.6 / 19 / 12 / 10	4.6
- Paarl	0.52 / 24 / 4.0 / 45	5.2
- Tygerberg	0.74 / 38 / 10 / 20	8.0
- Stellenbosch	0.76 / 20 / 5.2 / 105	3.2
Cape Agulhas	4.2 / 42 / 14 / 12	12
Overberg	2.4 / 26 / 9.4 / 10	7.2
Walker Bay	1.2 / 26 / 5.6 / 9	7.6
Western Cape**	0.48 / 44 / 5.4 / 153	7.2

** = all wine producing areas of the Western Cape.

Table 6.5.: IBMP levels (ng/L) in Sauvignon blanc wines from different wine-making regions for the vintages 2002-2006 (minimum / maximum / average / number of samples).

Region/district	2002	2003	2004	2005	2006
Breede River Valley					
- Breedekloof		2.6 / 20 / 7.0 / 10	4.4 / 26 / 11 / 7		
- Robertson			0.66 / 26 / 6.0 / 51		2.2 / 13 / 5.8 / 4
- Worcester	1.8 / 16 / 6.6 / 3	1.3 / 11 / 3.4 / 17	2.8 / 17 / 5.4 / 18		
Coastal Region					
- Constantia			12 / 18 / 15 / 3	6.6 / 20 / 11 / 12	
- Darling				13 / 19 / 15 / 6	
- Paarl			0.82 / 7.8 / 3.6 / 16	0.52 / 24 / 4.0 / 26	
- Tygerberg		0.74 / 14 / 5.2 / 5	3.6 / 13 / 8.8 / 4	6.4 / 12 / 9.6 / 7	
- Stellenbosch		3.4 / 5.6 / 4.4 / 4	0.76 / 20 / 4.8 / 30	1.0 / 16 / 5.4 / 66	2.8 / 10 / 5.4 / 3
Cape Agulhas				4.2 / 14 / 9.6 / 10	
Overberg			2.4 / 26 / 8.6 / 4	5.6 / 13 / 9.6 / 4	
Walker Bay				1.2 / 8.4 / 3.4 / 7	
Western Cape**		1.2 / 7.0 / 3.4 / 4	1.7 / 9.8 / 5.0 / 33	0.48 / 44 / 5.0 / 102	0.58 / 24 / 9.4 / 12

** = all wine producing areas of the Western Cape.

Maximum values for IBMP show some variance (17 to 44 ng/L) across the wine producing regions, and over the sampled vintages. The minimum, maximum and average values obtained for those samples in this investigation for which a specific regional delimitation were available, are graphically depicted in Figure 6.4. The maximum value was displayed in black, and at the centre of the three parameters in this bar graph to clearly illustrate variability across the regions.

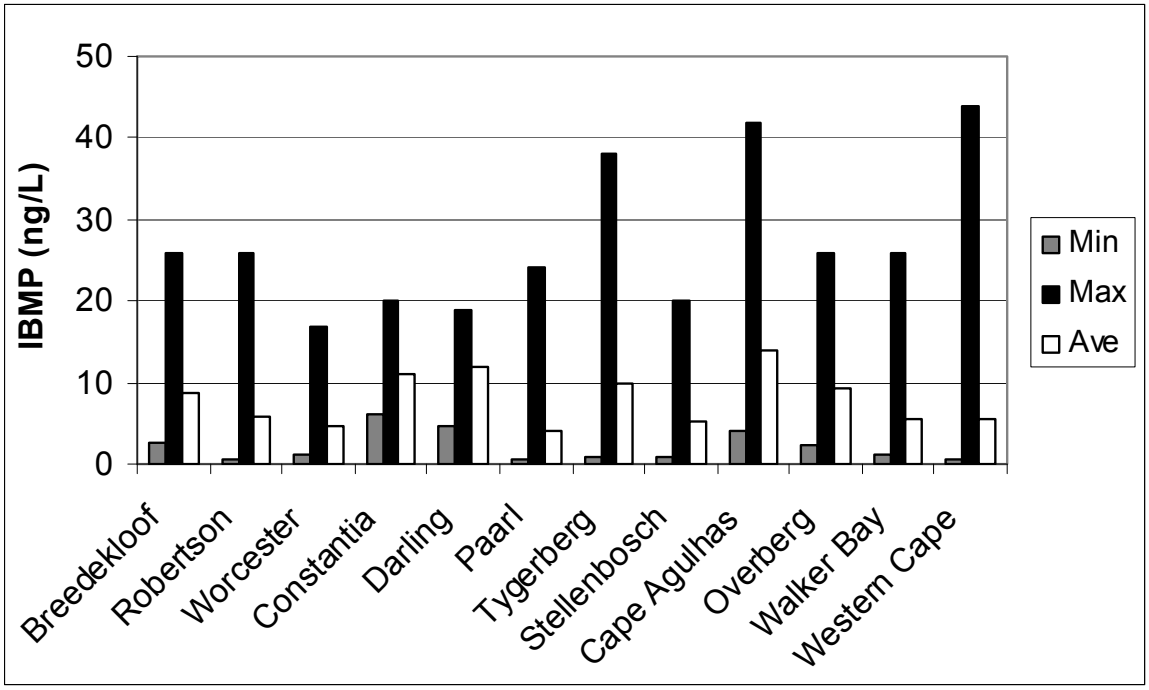

Figure 6.4.: Summary of the minimum, maximum and average values of IBMP in Sauvignon blanc wine as a function of region. Only samples with specific regional classification are shown, vintages 1999 to 2007.

From the information in Figure 6.4. it can be observed that some regions such as Constantia and Darling (cooler climate, close to ocean) are characterized by relatively high average IBMP values but that their maximum values are nevertheless lower than those of some warmer (inland) regions like Robertson and Paarl (which in turn had lower average values). On the other hand, Cape Agulhas, a cool region in close proximity to the ocean displays a relatively high average and maximum value. This variable nature of the occurrence of IBMP in South African Sauvignon blanc wines may be illustrated at the hand of two examples. Figure 6.5. presents an illustration of the IBMP content of wines produced on the same estate in the

Tygerberg district for seven consecutive vintages (1999 to 2005). The IBMP content varies between 6.4 and 38 ng/L (60% RSD).

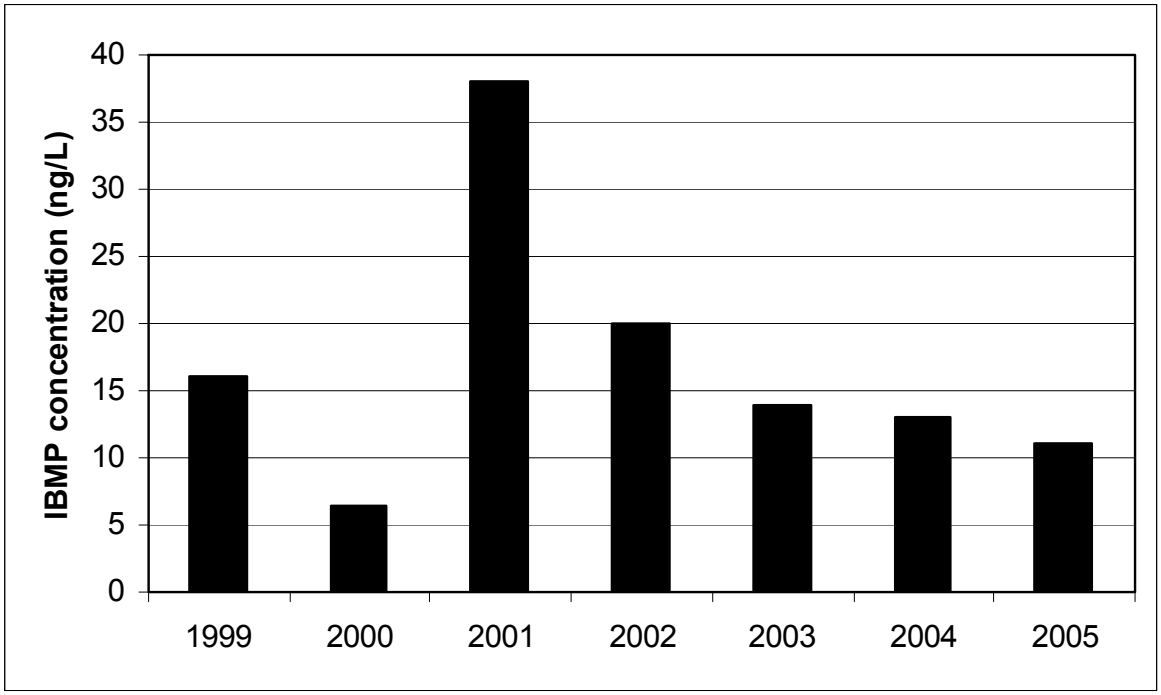

Figure 6.5.: IBMP content of Sauvignon wines produced on the same estate over seven consecutive vintages (1999 to 2005).

Significant variability of IBMP levels is also noted for wines that were produced during the 2004 vintage in the Stellenbosch district, on five adjacent estates, located approximately 8 km apart (Figure 6.6.). IBMP concentrations ranging from 2.1 to 11 ng/L were measured (73% RSD).

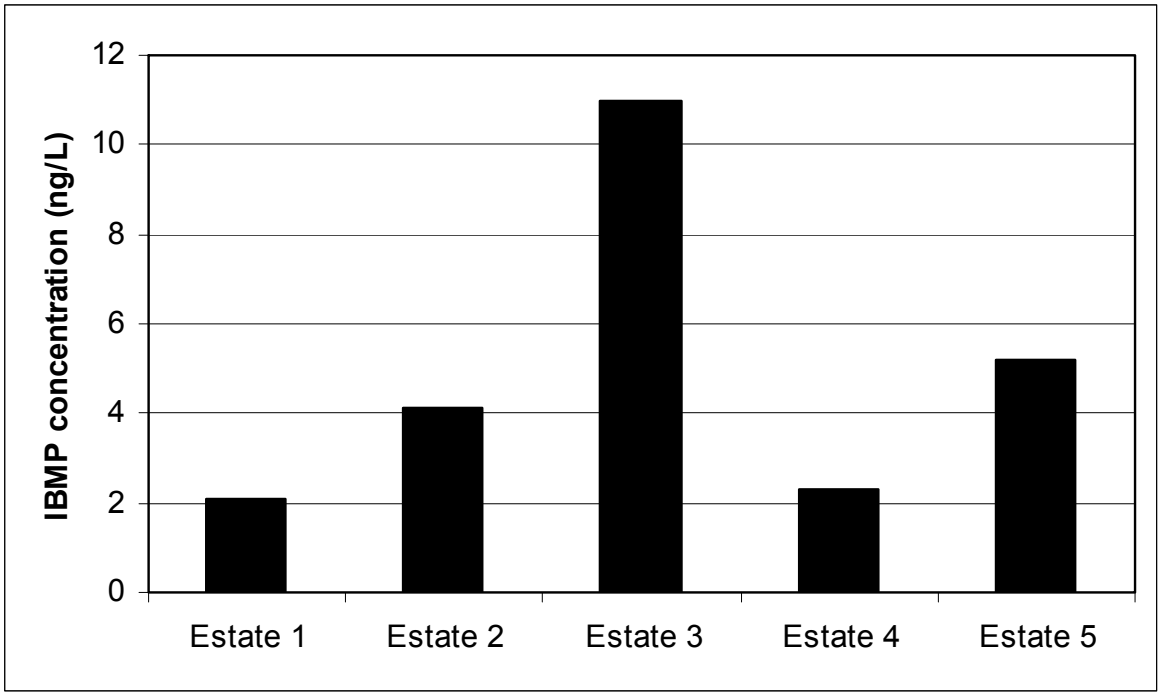

Figure 6.6.: IBMP content of Sauvignon wines (Stellenbosch, 2004 vintage) produced on five different estates, located in close proximity.

Considering the two illustrations presented above, it is clear that no rigid maximum values can be set for the concentration of IBMP in South African Sauvignon blanc wines. The data also suggest that factors such as the region of origin and vintage are not exclusively determinative regarding the IBMP content of the samples but that other factors, such as production variables may also play a role in determining the concentration, an observation supported (to some extent) by some local research.[5,10] The regional data in Table 6.4. seems to reinforce this observation as there is clearly no obvious correlation between maximum and average values between the regions, which would indicate that a particular region produces consistently high values. Cape Agulhas is an exception, producing a high average and high maximum value. The significance of this is that although a particular region may posses a low average, a relatively high maximum value may at the same time be expected, a point that is well illustrated by the data for the Paarl region (Figure 6.4.).

Despite this conclusion, the level of IBMP in wines across all vintages and geographical origins did not exceed 44 ng/L. Measured values above ~70 ng/L can

therefore be considered suspect, even though unambiguous identification of adulteration cannot be obtained by this criterion alone.

6.3.1.4. Determination of the relative ratios of abundance of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wines

Various authors reported tentatively that the relative amounts of three 3-alkyl-2-methoxypyrazines (IBMP, IPMP and SBMP) show a degree of consistency in some wines. [7,9] In this study IPMP and SBMP were determined in addition to IBMP. The concentrations of IPMP and SBMP in South African Sauvignon blanc wines were consistently lower than that of IBMP, and in some instances these values were below the LOQ (0.10 ng/L) of the method. It should be noted however that the analytical method used provides lower detection levels for all compounds compared to methods reported in the literature, and as such provides the most complete data for these compounds in Sauvignon Blanc wines to date. The ratio of IPMP:IBMP and SBMP:IBMP were determined where the former two compounds were quantified and the results expressed as a percentage relative to the IBMP concentration. Table 6.6. presents the details of the relationship between these compounds. It was found that the minimum ratios of IPMP:IBMP and SBMP:IBMP were 1%. Similar to the findings of Lacey *et al.* [7] these ratios show less variation than the overall IBMP concentration.

Table 6.6.: Statistics describing the ratios of relative abundance of the three quantified methoxypyrazines. Only wines where the said compounds were present above the quantification limit (0.10 ng/L) are presented.

	Ratio of IPMP:IBMP	Ratio of SBMP:IBMP
Minimum (%)	1	1
Maximum (%)	20	27
Average (%)	6	5
n	392	492
RSD (%)	46	67

On average, the two minor 3-alkyl-2-methoxypyrazines are present at levels of approximately 5% of the IBMP concentration. A number of possible explanations for

the variation in measured abundances may be found. The fact that South African producers may (legally)[1] add certain undisclosed amounts of wines from other cultivars to wine labelled as Sauvignon blanc may certainly contribute to the said variability. The variation of these ratios may also be attributed to different rates of evolution of the relevant species in the grape prior to harvesting, a phenomenon reported to occur in Cabernet Sauvignon grapes.[12] The time of harvest may then play a determinative role in the relative abundances.

The relatively large variation in these ratios indicates that no rigid values exist for the quantitative relationship between the three 3-alkyl-2-methoxypyrazines. A value of 1%, relative to IBMP, may however tentatively be considered as the minimum level and lower ratios may indicate possible adulteration of the wine sample with a source rich in IBMP. Such cases may then be evaluated by also taking into consideration the relative IBMP level of the sample.

Sauvignon blanc leaves were also analyzed in an attempt to determine the ratio between these components and as the 3-alkyl-2-methoxypyrazines probably partially originate from the leaves this may possibly provide useful information pertaining to the relevant abundances.[13] Leaves were sampled at two stages of development, darker green from the top of the vine and smaller light green (young) leaves from lower down. The samples were collected on the same day in October during the 2007 season. As the leaves were at a relatively early stage of development, the concentrations were relatively low with the result that only IBMP were quantified. The dark green leaves contained 44 ng/kg IBMP and the lighter leaves 32 ng/kg. Due to time constraints it was not possible to follow the evolution of the components of interest in the leaves.

6.3.1.5. Determination of the effect of adulteration on the 3-alkyl-2-methoxypyrazine profile

Several green peppers were analyzed as described in the experimental section to determine the relative amounts of 3-alkyl-2-methoxypyrazines, with the aim of determining the effect of wine adulteration with pepper extracts. These peppers

were at various stages of maturity: three were green, and one each yellow and red. Two locally marketed synthetic green pepper preparations were also analyzed for the target substances. These were labeled as “red bell pepper” and “red pepper” respectively. Table 6.7. presents the results obtained for green peppers and synthetic preparations.

Table 6.7.: 3-Alkyl-2-methoxypyrazine content of green peppers and synthetic preparations.

Sample	IBMP (ng/kg)	IPMP (ng/kg) / ratio	SBMP (ng/kg) / ratio
Green pepper	54 000	30 / 0.1%	110 / 0.2%
Green pepper	64 000	70 / 0.1%	380 / 1%
Green pepper	78 000	150 / 0.2%	530 / 1%
Green pepper (red)	42 000	2.4 / 0.01%	-
Green pepper (yellow)	44 000	20 / 0.1%	60 / 0.1%
Synthetic red bell pepper	54 000 000	-	5 500 000 / 10%
Synthetic red pepper	192 000 000	90 000 / 0.05%	24 000 / 0.01%

It is clear that green peppers contain significantly higher amounts of IBMP, relative to IPMP and SBMP, than is the case for Sauvignon blanc wines. IBMP represent approximately 99% of the total 3-alkyl-2-methoxypyrazine content of green peppers. This observation correlates exactly with the findings of Murray *et al.* regarding the ratios of these compound in green peppers.[8] IBMP was also the dominant congener in the two synthetic preparations that were available. The product labeled as “red pepper” displayed the same approximate ratios as the fruit, whereas IPMP was absent from the product marked as “red bell pepper” and SBMP was present at approximately 10% of the abundance of IBMP. It is therefore clear that adulteration of wine with either green peppers or synthetic preparations may enrich the levels of IBMP disproportionately, thereby altering the ratio of this compound relative to IPMP and SBMP.

6.3.1.6. Adulterated Sauvignon blanc wines

A few samples in the investigation were previously tentatively identified as possible cases of adulteration based upon sensorial evaluation of these products while two samples were known to be adulterated following admissions from the producer in

this regard.[2] These two known adulterated wines were identified as such during the course of the investigation. These wines contained 310 and 960 ng/L of IBMP respectively, exceeding the highest value (44 ng/L) found in the study by a factor of 7 and 22, respectively. For convenience these samples will be referred to in the following discussion as adulterated sample 1 and adulterated sample 2. A graphical representation of the levels of IBMP found in the study, including the adulterated wines, is presented as Figure 6.7. The adulterated samples clearly contain excessive levels of IBMP relative to the sample population.

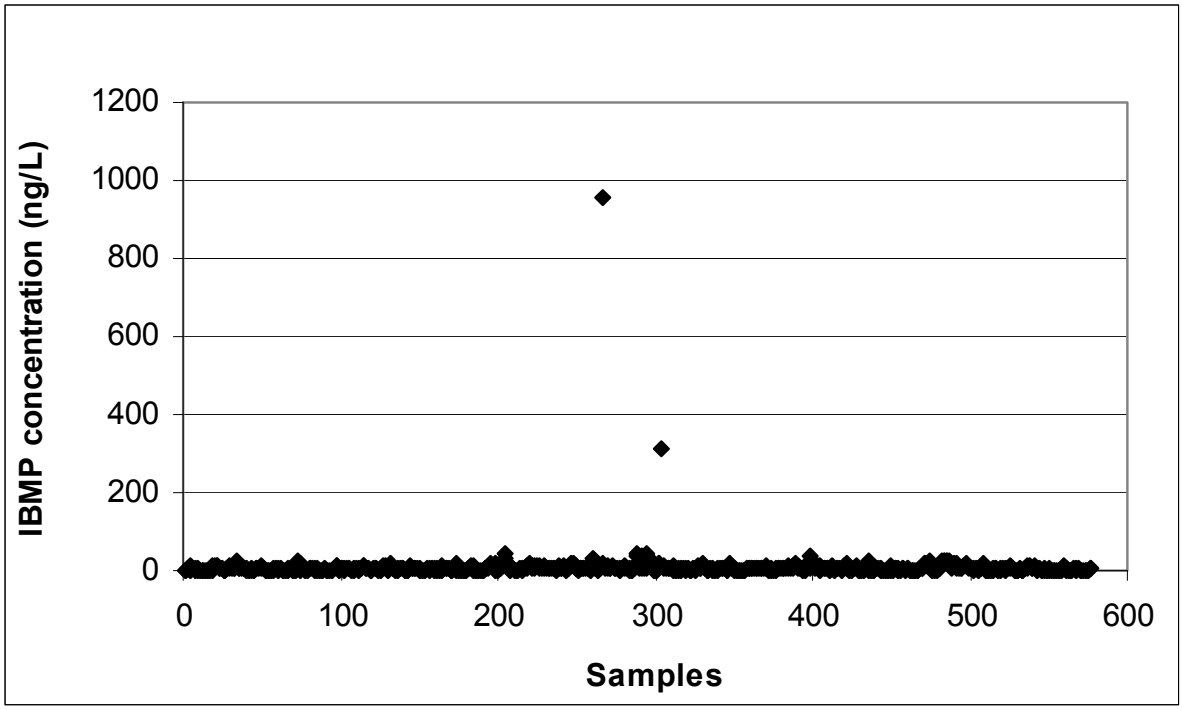

Figure 6.7.: IBMP content of all analyzed Sauvignon blanc samples.

The Dixon Q-test was used to prove that the IBMP content of the two adulterated samples differs significantly from the rest of the samples and that they are therefore outliers. In the Dixon Q-test the absolute value of the difference between the outlier (x_q) and its nearest neighbor (x_n) is divided by the spread of the entire set (w) to give the quantity Q_{exp}. This ratio is then compared with a set of rejection values (Q_{crit}) at various levels of confidence. If Q_{exp} is greater than Q_{crit}, the suspected value may be considered as significantly different from the rest of the set with the indicated degree of confidence.[1,14] It should be noted that in the following calculations the two outliers

were considered separately: adulterated sample 1 was not considered to be part of the set when Q_{exp} were calculated for adulterated sample 2 and vice versa.

Q_{exp} is given by: $Q_{exp} = | x_q - x_n | / w$

Table 6.8.: Result obtained for the Dixon Q-test for the two adulterated Sauvignon blanc samples.

Sample	x_q	x_n	w	Q_{exp}	Q_{crit} at 99% confidence
Adulterated sample 1	310 ng/L	44 ng/L	309.6 ng/L	0.859	~ 0.372 (n = 30)
Adulterated sample 2	960 ng/L	44 ng/L	959.6 ng/L	0.955	~ 0.372 (n = 30)

From the data summarized in Table 6.8. it is evident that the IBMP content of the two adulterated samples are significantly different from the rest of the samples (n = 575) analyzed in the current investigation. The Q-test rejection criteria were based on a 99% degree of confidence and for 30 observations. It may therefore be concluded that the IBMP concentration of the said samples were enriched artificially as the natural abundance was exceeded by a significant margin.

The ratios of IPMP and SBMP relative to IBMP in the two adulterated wines were determined to be very low (Table 6.9.). These ratios are consistent with adulteration with green pepper extracts or some synthetic preparations resulting in disproportional enrichment of the IBMP content of the samples.

Table 6.9.: Ratios of relative abundance of the three analysed methoxypyrazines for the two adulterated wines compared to the other wines.

	Ratio IPMP:IBMP (%)	Ratio SBMP:IBMP (%)
Minimum (all other samples)	1	1
Average (all other samples)	6	5
Adulterated sample 1	0.2	0.04
Adulterated sample 2	0.07	0.01

Due to the large spread of these values (1 to 20% and 1 to 27%), the Q-test could not be used to prove that the values for adulterated sample 1 and adulterated sample 2 were not part of the subset of natural abundances. The observed ratios for

the two adulterated wines were however consistently lower than the minimum values for all other samples by a factor of between 5 and 100. These results indicate that the absolute level of IBMP serves as a more useful criterion for the detection of adulterated wines than do the ratios of the principle methoxypyrazines, at least in the case where wines are spiked to the extent observed for the two adulterated samples analysed here.

6.3.2. 3-Alkyl-2-methoxypyrazine content of other cultivars

The aim of the study was to identify adulteration of Sauvignon blanc wines through investigation of the 3-alkyl-2-methoxypyrazine content of the product. As the method produces comprehensive information on these compounds in wine, several wines of different cultivars were also analyzed in order to obtain a wider perspective of the occurrence of these substances in South African wines. As the levels in most of these products were relatively, low only IBMP were quantified here. Table 6.10. shows the results obtained for wines of other cultivars (various vintages and origins).

Table 6.10.: Quantitative data for IBMP for wines of other cultivars.

Cultivar	n	Min. (ng/L)	Max. (ng/L)	Ave. (ng/L)	Stdev. (ng/L)
Pinotage	6	0.78	3.1	1.5	0.86
Chardonnay	6	0.20	1.0	0.53	0.28
Cabernet Sauvignon	6	3.4	17	10	4.8
Shiraz	6	1.5	3.4	2.4	0.62
Chenin blanc	4	0.38	1.1	0.59	0.35
Merlot (experimental)	14	26	56	42	10
Merlot (commercial)	2	6.9	7.7	7.3	0.57

As was the case with Sauvignon blanc wine, IBMP values (wines of other cultivars) were higher in French wines. Values for IBMP in French Cabernet Sauvignon varied between 5 and 30 ng/L with an average of 18 ng/L. French Merlot wine contained between 4 and 23 ng/L IBMP with an average of 12 ng/L.[11] Very low levels of the other 3-alkyl-2-methoxypyrazines (IPMP and SBMP) were correspondingly reported for French Cabernet Sauvignon wine. Allen et al. found that the concentration of SBMP did not exceed 2% of that of IBMP.[9]

No information was obtained for other cultivars in Table 6.10. Pinotage is a cultivar that is unique to South Africa and to the best of our knowledge these compounds have never been detected in these wines. The experimental Merlot samples produced very high levels relative to the other South African wines. The experimental Merlot wines were produced from a single experimental vineyard where the effect of different viticultural practices was studied. Although a detailed evaluation of these parameters on the IBMP content of the wines falls outside the scope of the current work, it can be noted that differences in viticultural practices have a significant effect on the IBMP content (a factor two for the same vintage, vineyard and clone in this example).

6.3.3. Multivariate analysis of the 3-alkyl-2-methoxypyrazine data

A number of multivariate data analysis techniques exists that may be utilized to extract useful information from complex data sets. The key to obtain the desired information is to establish the objective of the investigation clearly. Multivariate data analysis (MVA) may be used to achieve three main aims, namely: data description, discrimination and classification, and regression and prediction.[15] In this study principal component analysis (PCA) was utilized to study the differences between wine samples (discrimination) in the methoxypyrazine dataset. The objectives of the PCA study were to confirm the conclusions regarding the adulterated wines as well as to study possible correlations between the data and vintage and region of origin of the products. The latter was attempted because for such a large dataset containing many variables, it is not easy to visualize such correlations.

6.3.3.1. Brief overview of PCA

PCA involves transformation of a set of correlated variables to a set of uncorrelated factors (referred to as principal components or PC's) in such a manner that the underlying covariance is maximized.[15] The utility of PCA stems from the fact that the derived variables are often better descriptors of the data structure than the original

variables. As a large part of the variance in the original dataset can be modeled by a limited number of latent variables, PCA effectively reduces the dimensionality of the data and in this way facilitates visual inspection of complex data sets.[15,16] The results of PCA are therefore commonly interpreted visually. As the total variance within the data set is mainly accounted for by the first few PC's, these may be used plot the data. The similarity or differences between objects (samples) may then be determined readily from these plots.

The original variables are related to the PC's, and loading vectors, also called loadings, may be viewed as the bridge between these entities. Loadings reveal the extent of the contribution of each variable to meaningful variation in the data.[15] Therefore the loadings of each of the original variables plotted against the derived latent variables produces a loadings plot that may be used to identify those variables responsible for differentiation of samples on each PC. From the loadings plot the correlation (i.e. co-variance) between variables may also be determined, with the angle between loading vectors indicative of their correlation. This is used to study potential relationships between the original variables. On the other hand, a scores plot depicts the scores of each object or sample on selected PCs, and is used to study the relationships between samples. Distinct clusters would reveal the grouping of similar objects. Typically, scores plots are used to investigate potential grouping of samples, while subsequent investigation of loadings plots can be used to determine which variables are responsible for this differentiation between samples.

6.3.3.2. Results for adulterated wines

PCA was first applied in order to corroborate results for outliers (adulterated samples 1 and 2, numbered 18 and 19) with those obtained by univariate statistics. To investigate the two outliers PCA was performed using only IPMP, IBMP and SBMP variables. The loading plot for PC1 and PC2 (Figure 6.8.) reveals that IPMP and SBMP are positively correlated with PC1 while IBMP is likewise correlated with PC2. The loading vector for IBMP is approximately orthogonal to those of IPMP and SBMP, indicating that these variables do not covary. The IBMP content therefore plays a minor role in discriminating wines according to PC1 and vice versa. The

score plot clearly show the two adulterated samples differentiated on PC2. These two samples are therefore distinguished based on their (high) IBMP content. Sample 96 is distinguished on PC1, based on a high IPMP and SBMP content. This product is a barrel-fermented Sauvignon blanc wine, indicating that this wine style may possibly be correlated with high ratios (IPMP:IBMP and SBMP:IBMP) as values of 13 and 16% were obtained, compared to mean values of ~5%. Regarding the following figures, it should be noted that the term “factor” (Statistica) refers to the principal component (PC).

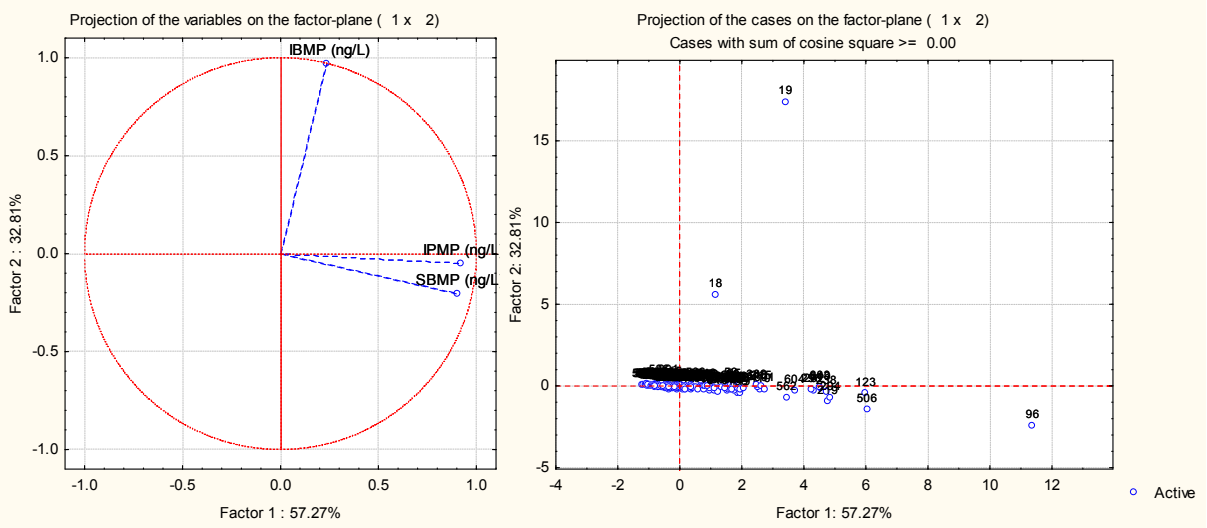

Figure 6.8.: PCA analysis to discriminate adulterated samples, only IPMP, IBMP and SBMP variables.

6.3.3.3. Grouping of wine samples according to geographical origin

It has been hypothesized that region of origin plays an important role in determining the 3-alkyl-2-methoxypyrazine content of Sauvignon blanc wines. Quantitative differences were reported between French, New Zealand and Australian products[6,7] as well as between locally produced wines, with different origins[5,10]. Local investigations attributed regional differences in IBMP concentration to macro- and microclimatic variations between regions and seasons.[10] PCA and Anova were used to investigate possible grouping of samples according to origin.

Grouping of wine samples according to origin and the possible existence of a relationship between 3-alkyl-2-methoxypyrazine content and other wine parameters (alcohol and reducing sugar content, total acidity and pH) were investigated using PCA with the objective to use this information for authenticity elucidation. The two outliers (adulterated sample 1 and 2) and samples with non-specific regional classification (such as Western Cape), were removed from the dataset. Firstly all variables were used (IPMP, IBMP and SBMP concentration as well as alcohol and reducing sugar content, pH and total acidity) and then secondly only the methoxypyrazine variables. The loading plot shows that in PC2 total acidity (TA) and pH are inversely related (as expected, high acidity leads to low wine pH). PC1 accounts for 35% of the variance, and the methoxypyrazines are responsible for variation explained by this PC. Significant correlation (covariance) between the three methoxypyrazines are observed on PC1 (Figure 6.9.).

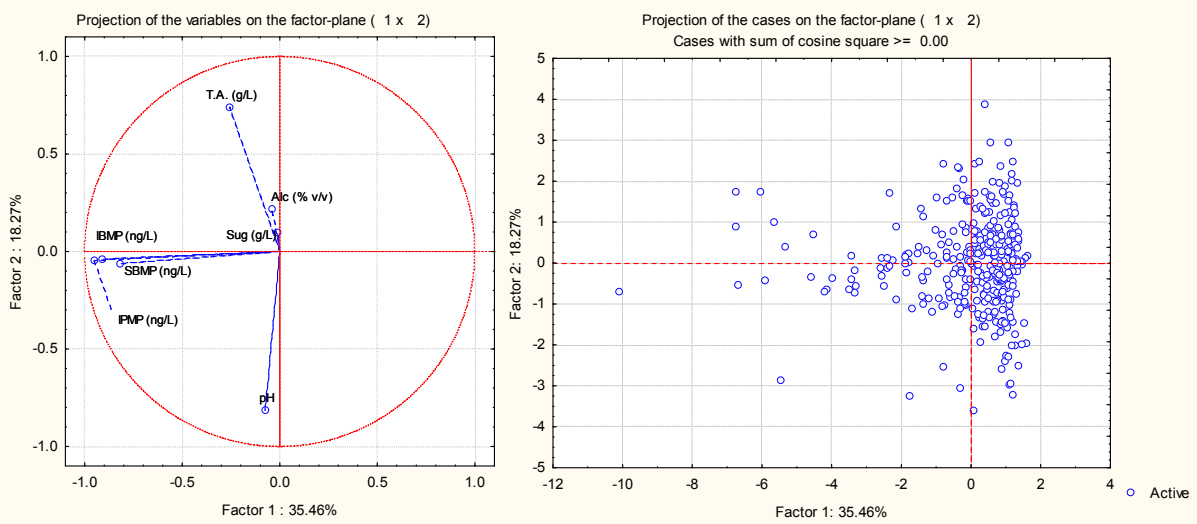

Figure 6.9.: PCA analysis for grouping of samples according to region of origin, all variables included (PC1 versus PC2).

No clear distinction according to region is evident from the score plot although some samples are differentiated based on high methoxypyrazine content and are positioned to the left in Figure 6.9. PC1 versus PC3 distinguishes a single wine from Constantia but this separation is based on an unusually high sugar content for this noble late harvest wine (Figure 6.10.). Omission of this sweet Constantia wine from the relevant dataset yielded no significant improvement in the separation (not shown).

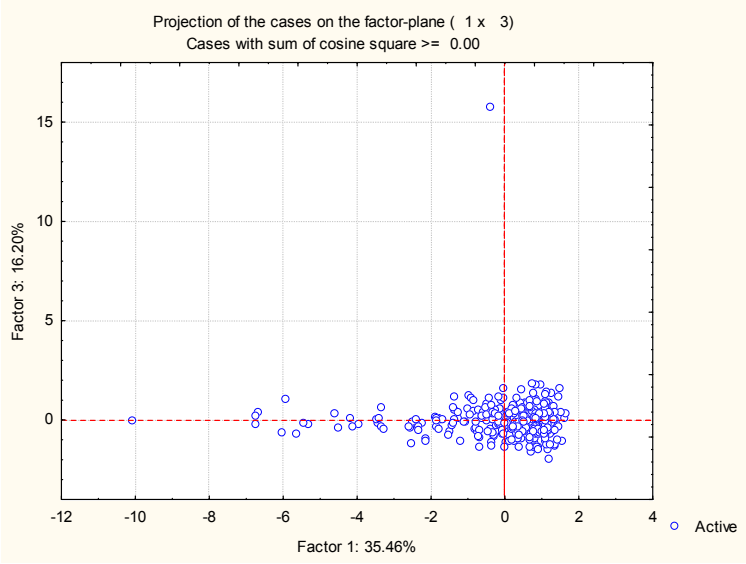

Figure 6.10.: PCA analysis for grouping of samples according to region of origin with all variables included, PC1 versus PC3.

The same analysis was then performed but using only three variables (IPMP, IBMP and SBMP). Here PC1 accounts for variation in overall methoxypyrazine content while in PC2 IBMP and IPMP are inversely correlated to SBMP (Figure 6.11.).

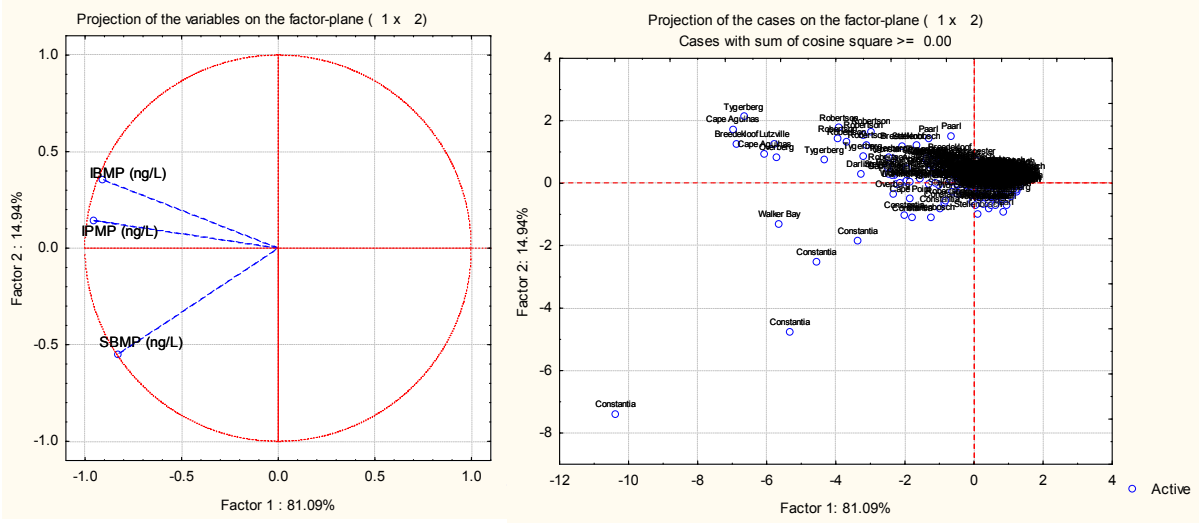

Figure 6.11.: PCA analysis for grouping of samples according to region of origin, only IPMP, IBMP and SBMP variables, PC1 versus PC2.

Apart from partial separation of cooler climates wines (Constantia and Walker Bay), no clear distinction was once again obtained with PCA as was the case with a plot of PC1 versus PC3 (not shown).

Anova was also performed to investigate the methoxypyrazine data. A prerequisite for reliable Anova is that the samples display a Gaussian distribution of values and an equal representation within different groups. Due to sample availability constraints these requirements were not fulfilled in all instances. As some origins were less well represented than others, the bootstrap correction was applied to the data for Anova to correct for the skew distribution of the samples. Anova indicated that significant differences ($p < 0.05$) exist between wines of some origins (e.g. between Stellenbosch and Overberg, Cape Agulhas, Constantia and Darling), but that methoxypyrazine concentration can not be used for rigorous classification of origin using the current data set. Figure 6.12. shows the means and 95% confidence intervals (corrected) for IBMP concentrations from different regions. Similar trends were observed for IPMP and SBMP (results not shown).

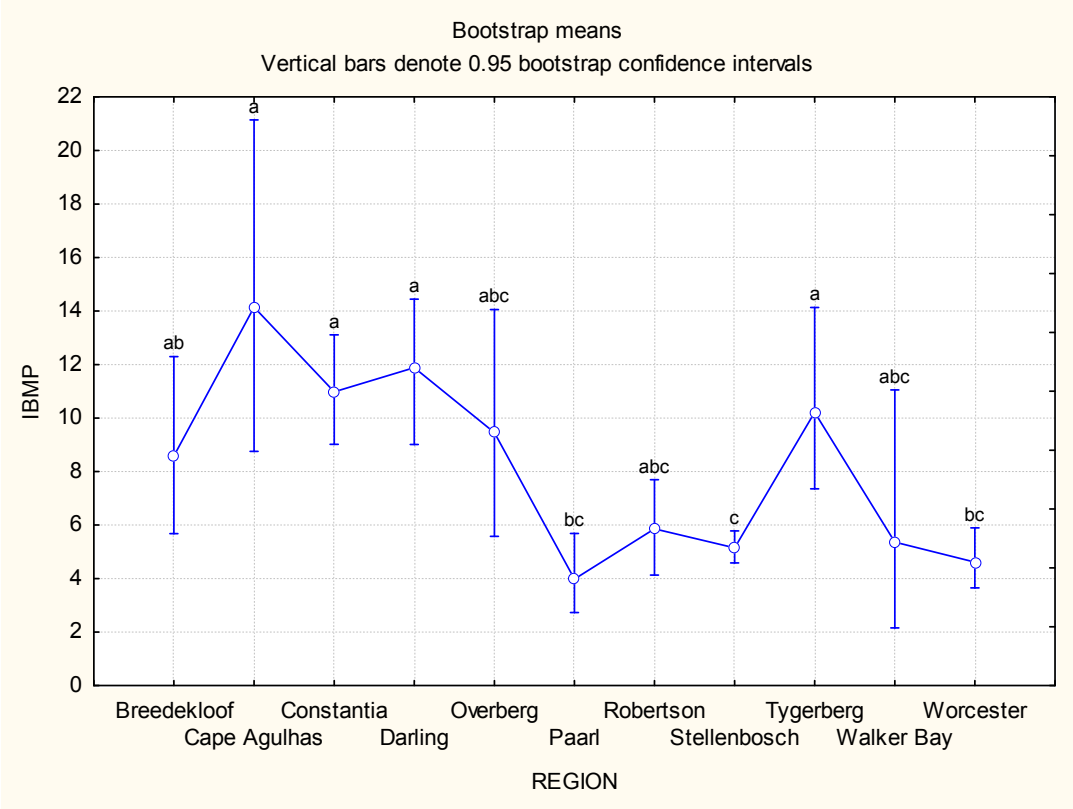

Figure 6.12.: Anova results for wine samples according to region of origin using IBMP concentration (means and 95% confidence intervals, bootstrap corrected).

For the following figures only two distinct regions were compared (Constantia (C) and Worcester (Wo)), as these regions are relatively cool and warm respectively and more pronounced differences are therefore expected.

The loading plot for all variables reveals that the pyrazines contribute almost exclusively to PC1 while the other variables contribute mainly to PC2 (Figure 6.13.). TA and pH are also directly related here as opposed to before (with all regions). The spreading of Constantia (C) wines to the right on the score plot are due to higher methoxypyrazine values while the one sample distinguished on PC2 is due to the high sugar content of that sample (noble late harvest wine). When the analyses was performed on the same subset, but with the two extreme C-values omitted, a slight but inconclusive improvement was observed (results not shown).

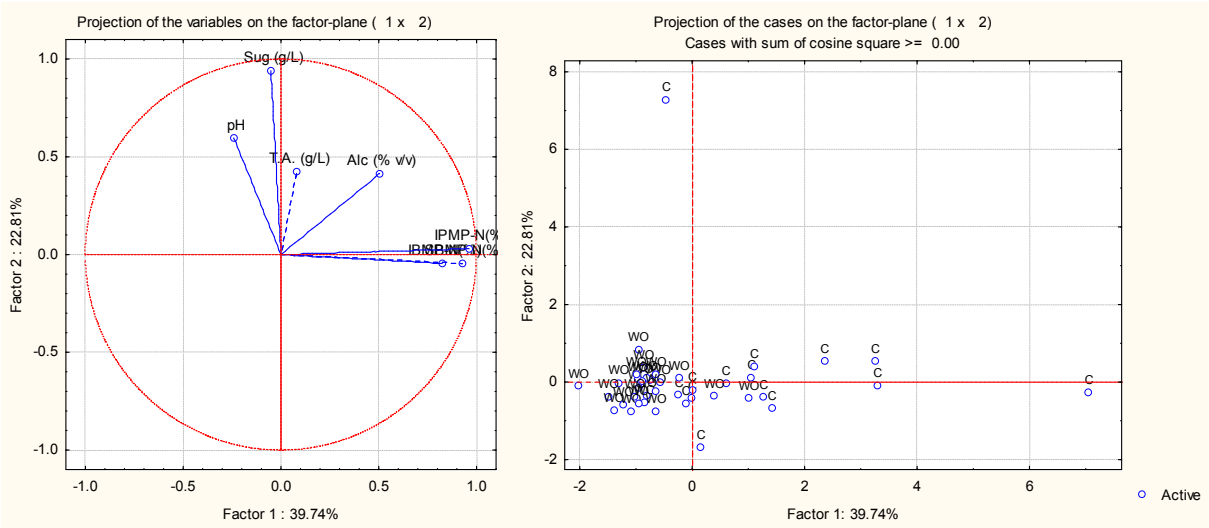

Figure 6.13.: PCA analysis for grouping of samples according to region of origin (only wines from Constantia (C) and Worcester (Wo) included), using all variables, PC1 versus PC2.

Similar differentiation is observed on the score plot of PC1 versus PC3, mainly due to differences in methoxypyrazines content (Figure 6.14.). Although no clear separation was achieved, it can however be accepted that Constantia (C) produces Sauvignon blanc wine with slightly higher methoxypyrazine concentrations.

Figure 6.14.: PCA analysis for grouping of samples according to region of origin for samples from Constantia (C) and Worcester (Wo), using all variables, PC1 versus PC3.

The same subset of samples was analyzed using only the three methoxypyrazine values for each sample. The loading plot now shows that the three variables are mainly correlated (negatively) with PC1 while the score plot displays slight differentiation of some Constantia wines, attributed mainly to this PC. Once again some differentiation is possible without complete separation (Figure 6.15.). PC1 versus PC3 produced similar results (not shown).

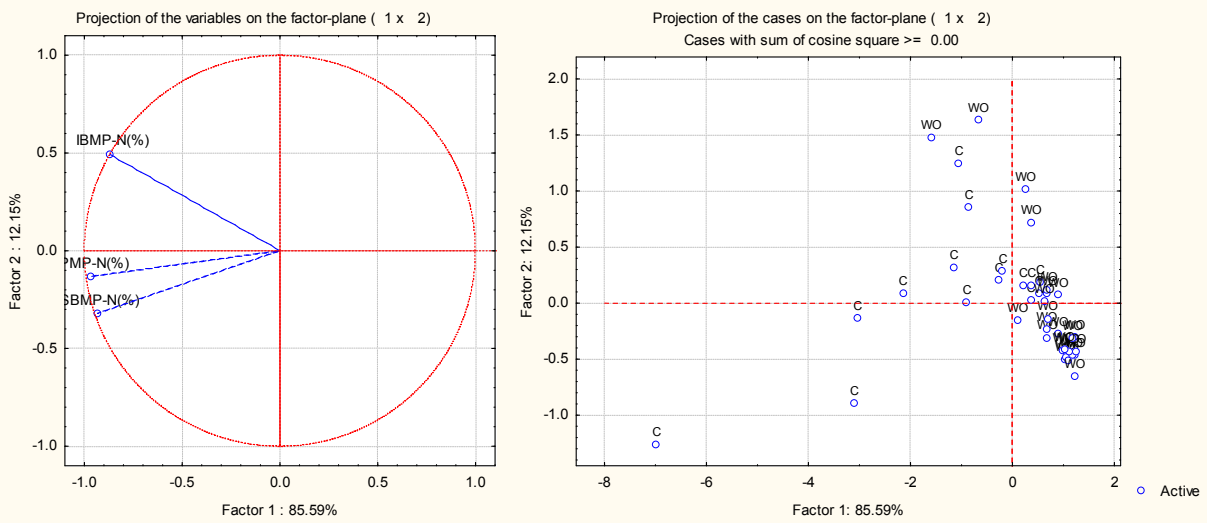

Figure 6.15.: PCA analysis for grouping of samples according to region of origin for samples from Constantia (C) and Worcester (Wo), using methoxypyrazine variables, PC1 versus PC2.

Anova revealed that significant differences exist between wines from Constantia and Worcester ($p < 0.05$). It should be noted though that the limiting factors discussed for Anova is also valid here due to sample availability constraints.

As these PCA results did not show clear separation of these two regions (Constantia and Worcester), the exercise was repeated using data for Stellenbosch (ST) and Paarl (P). The loading plot for all variables revealed the same trends as discussed before for Constantia and Worcester. The methoxypyrazines carry approximately the same weight in PC1 but almost none in PC2. No improved separation was obtained for these samples compared with the previous combination.

The loading plot of PC1 versus PC2 utilizing only the methoxypyrazine data revealed that the three pyrazines are negatively correlated (to the same extent) on PC1, while their loadings on PC2 were smaller (IBMP was positively, and IPMP and SBMP negatively correlated on this PC). A slightly improved separation was obtained with Paarl (P) confined mostly to positive PC1 and 2, possibly due to lower total methoxypyrazines with relatively higher IBMP. PC1 versus PC3 produced no additional information (Figure 6.16.).

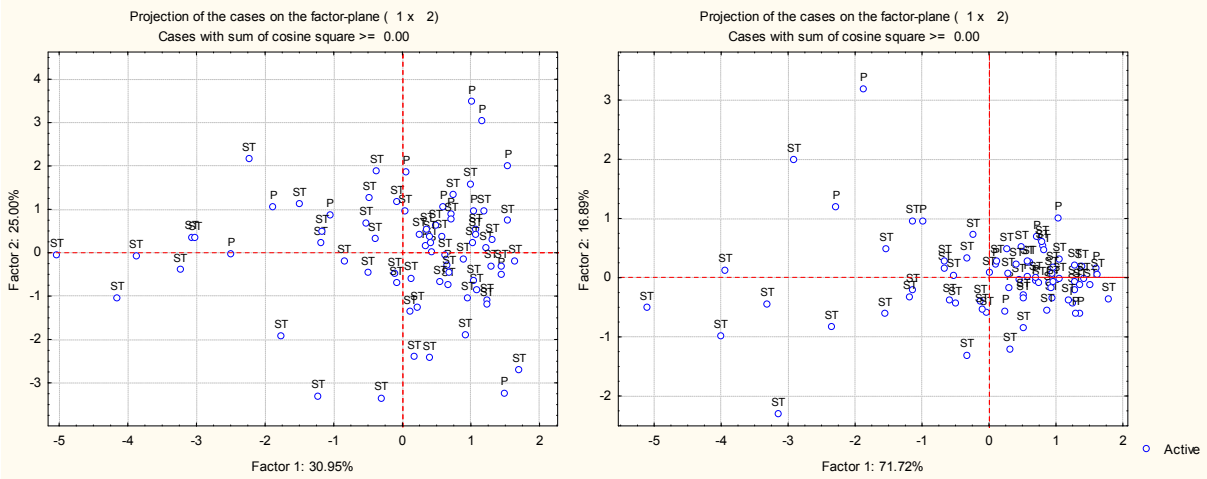

Figure 6.16.: PCA analysis for grouping of samples according to region of origin for samples from Stellenbosch (St) and Paarl (P), using all variables (left) and only methoxypyrazine variables (right). PC1 versus PC2.

Anova also showed that there was no significant difference between Stellenbosch and Paarl methoxypyrazine data ($p > 0.05$).

The same exercise was repeated for Robertson and Constantia without any clear differentiation between these regions with PCA (results not shown). Anova similarly reveal no significant difference between the data for Robertson and Constantia ($p > 0.05$).

6.3.3.4. Grouping of wine samples according to vintage

Vintage has been reported to play an important role in determining the methoxypyazine content of wines from a particular region, mainly through annual variations in average temperature and rainfall.[5] PCA and Anova were applied to the dataset in order to determine whether grouping of wine samples according to vintage could be observed. The dataset contained samples of vintages 2002 to 2006 and all variables were included initially. The loading plot once more revealed that PC1 carries most of the information and is positively correlated with methoxypyrazine content. Some wines from 2005 appear to be differentiated

according to high methoxypyrazine content but generally no clear differentiation according to vintage is obtained (Figure 6.17.).

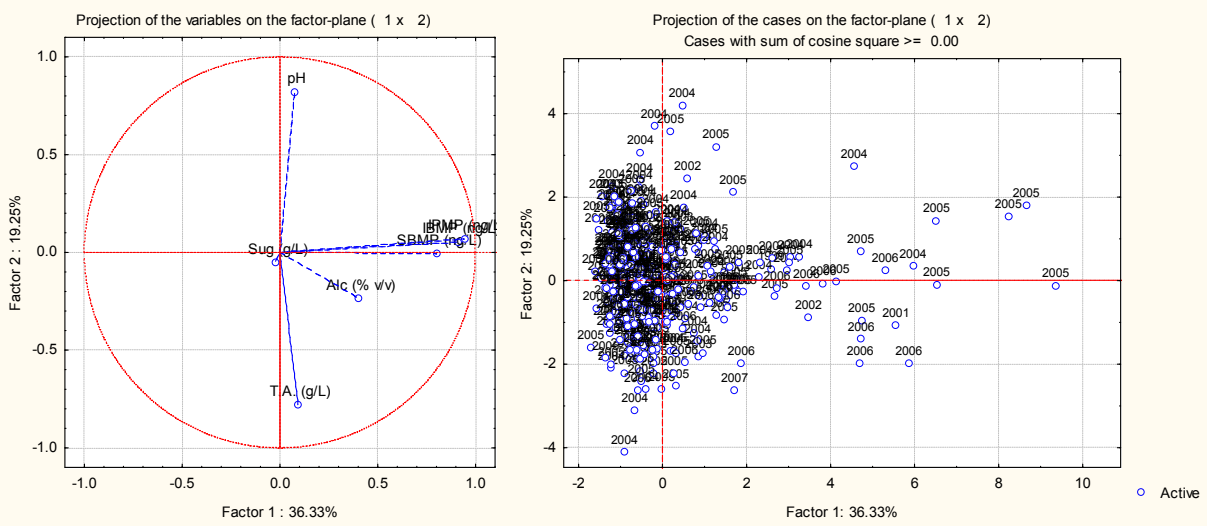

Figure 6.17.: PCA analysis for grouping of samples according to vintage, using all variables (PC1 versus PC2).

Anova (Figure 6.18.) showed that significant differences exist between some vintages when 2002 and 2006 were included ($p < 0.05$). However, Anova of the 2004 and 2005 data (197 and 295 samples, respectively) revealed no significant differences between these vintages ($p > 0.05$). As the subsets of samples of vintages 2004 and 2005 comply with the requirements for reliable Anova, it may be concluded that no significant differences between vintages were observed where reliable data was available. The same trends were also observed for IPMP and SBMP.

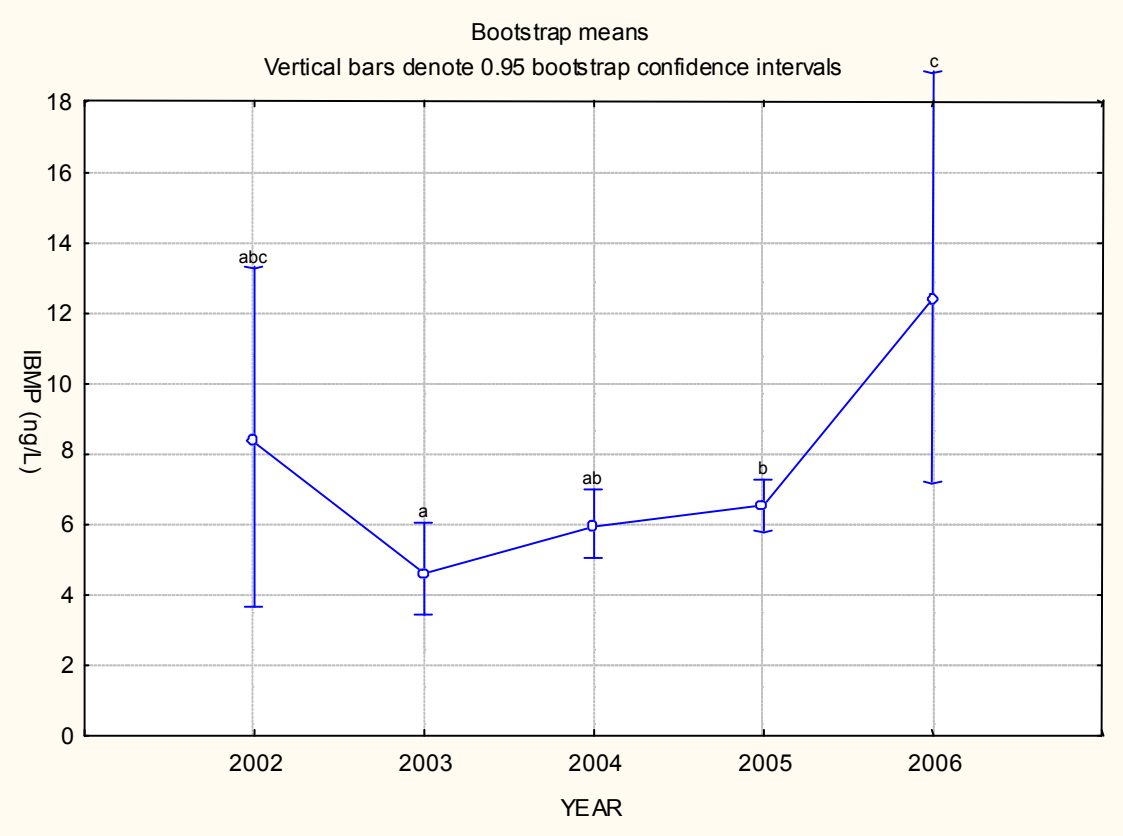

Figure 6.18.: Anova results for wine samples according to vintage using IBMP concentration (means and 95% confidence intervals, bootstrap corrected).

The exercise was then repeated utilizing PCA and using only the methoxypyrazine variables (Figure 6.19.). The methoxypyrazines were correlated negatively on PC1, which also carried most of the information. The score plot once again revealed limited separation based on higher methoxypyrazine values in the 2005 vintage.

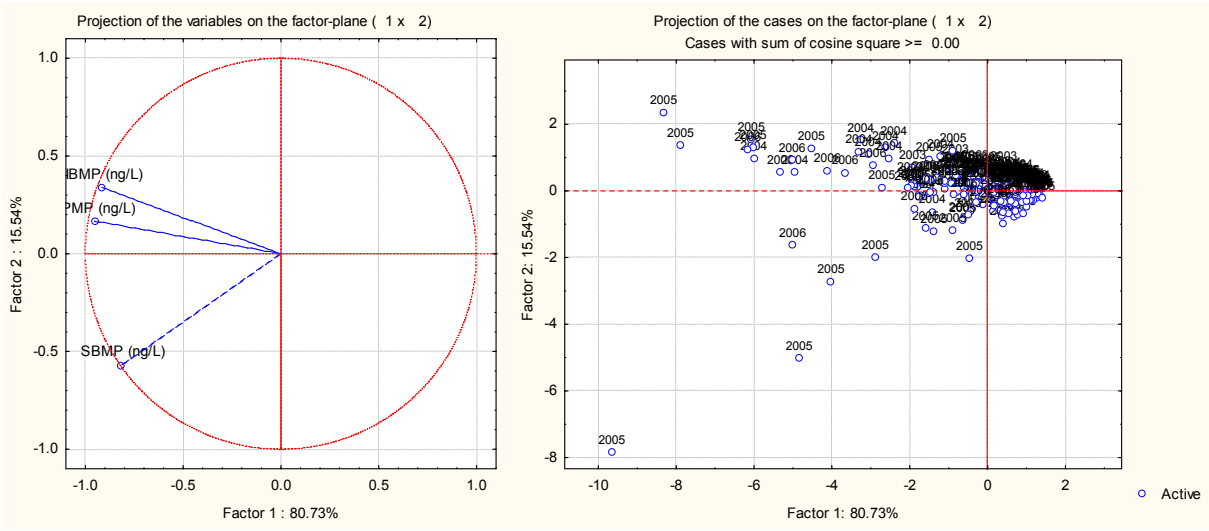

Figure 6.19.: PCA analysis for grouping of samples according to vintage, only methoxypyrazine variables used (PC1 versus PC2).

As this result produced a slight measure of success, this was investigated further by analyzing data for the Stellenbosch region only, attempting classification according to vintage (Figure 6.20.). No classification according to vintage was however achieved for the Stellenbosch region and a plot of PC1 versus PC3 (not shown) similarly lacked any clear structure.

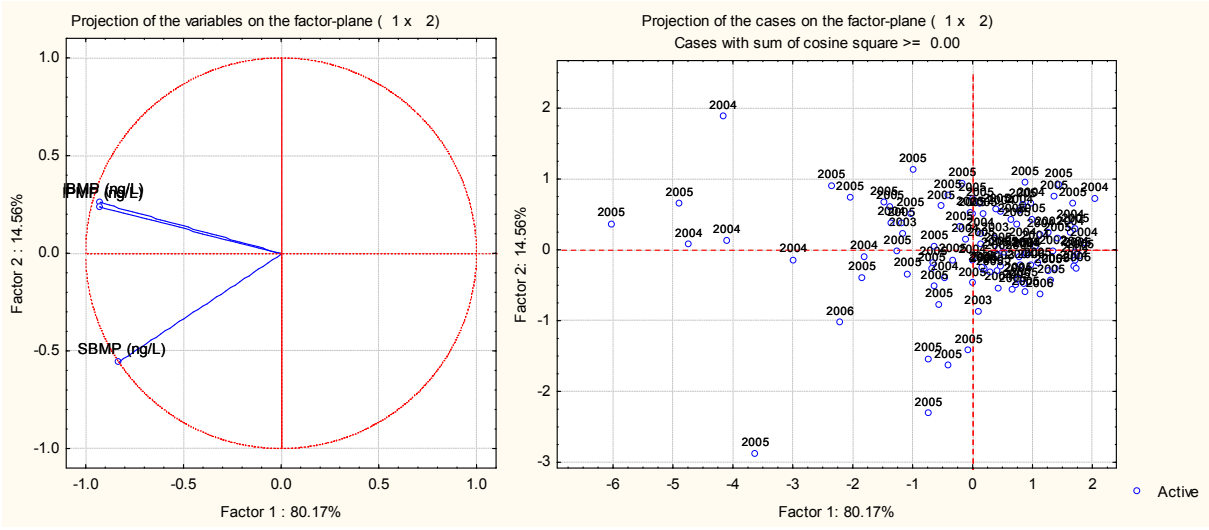

Figure 6.20.: PCA analysis for grouping of samples according to vintage, Stellenbosch region and methoxypyrazine variables only (PC1 versus PC2).

The same exercise was then repeated, attempting to classify data from various regions according to vintage, analyzing data from Constantia, Darling and Overberg.

The results as before were inconclusive, only data for Darling are displayed in Figure 6.21. as this is representative of the outcome of the other evaluations. The loading plot revealed the same correlation of the three methoxypyrazines with PC1.

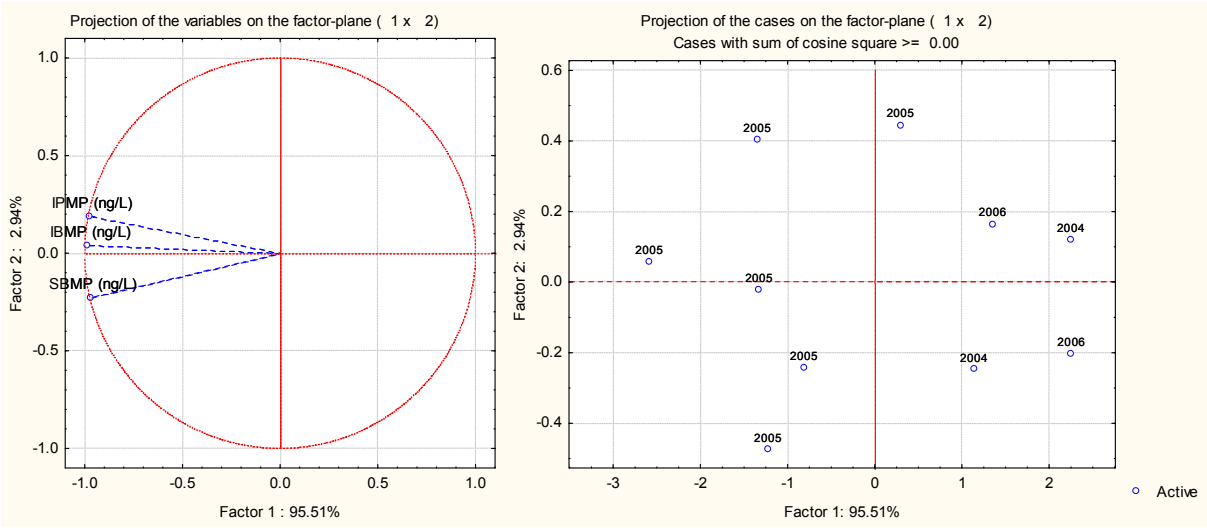

Figure 6.21.: PCA analysis for grouping of samples according to vintage, Darling region and methoxypyrazine variables only (PC1 versus PC2).

6.3.3.5 Conclusions for multivariate data analysis

The two known adulterated samples, identified as outliers using univariate statistics, were clearly differentiated by PCA on account of their exceptionally high IBMP content. The loading plot for the data subset containing these samples was unique as the loading vector for IBMP was orthogonal to that of IPMP and SBMP indicating that these variables do not covary, as was the case in all other instances. This fact implies that the IBMP concentration was not related to that of IPMB or SBMP. These two samples were clearly differentiated on PC2 as IBMP was positively correlated with this PC while the other two variables displayed small loadings for PC2.

Except for the sweet Constantia wine, the three methoxypyrazine variables accounted for most of the variation in the data set as they are consistently largely correlated with PC1. The ratios of relative abundance of the methoxypyrazines (IPMP to IBMP and SBMP to IBMP), contributed very little information as the three variables produced relatively similar loadings on PC1 while at the same time their

contributions to PC2 was poor. Clear differentiation of the samples according to region of origin utilizing PCA was not achieved. Considering that two extreme cases (Constantia and Worcester) were only partially differentiated illustrates that other production factors possibly play a far more determinative role in this regard. It was similarly found that clear differentiation of the samples according to vintage was not evident from PCA, even considering only samples form a specific region. Stellenbosch, Constantia, Darling and Overberg were unsuccessfully evaluated for possible differentiation according to vintage.

The loading plots for the data also revealed that the loading vectors for the three methoxypyrazines and the other variables (alcohol and sugar content, total acidity and pH) were in general consistently orthogonal indicating that the two sets of variable are unrelated. As no covariance was thus observed, the time of harvesting and phenological ripeness (that might manifest in a high total acidity for relatively early harvesting[4] and vice versa) does not appear to play a determinative role in the methoxypyrazine content of the samples. The same general observation also applies to the sugar content, which showed very little covariance with the methoxypyrazine content of the sample (here a high sugar content may indicate relative late harvesting resulting in lower wine methoxypyrazines).

It can be concluded that the significant variance in methoxypyrazine content and ratios cannot unambiguously be ascribed to either geographical origin or vintage.

6.4. Conclusions

Two wines were identified as adulterated based on criteria that were developed in this study. The Dixon Q-test test was applied to the data pertaining to the IBMP concentration of the samples to demonstrate that the outliers were significantly different from the population and that the deviations observed cannot be ascribed to natural variance within the dataset. The ratios of abundance of IPMP and SBMP relative to IBMP in the two adulterated products were also significantly lower than those of the unadulterated samples. Although this difference could not statistically be classified as significant, this phenomenon is consistent with adulteration of wine

with green pepper extracts or synthetic preparations. Results for the two adulterated wines were confirmed utilizing PCA. The PCA loading plot revealed that with the two adulterated wines, IBMP did not covary with IPMP and SBMP indicating that the IBMP content in these samples were disproportionally high compared to unadulterated samples.

Investigation of the data by PCA and Anova indicates that no clear grouping of wines according to vintage or region of origin is evident. Knowledge of the vintage or region of origin therefore provides very limited information for elucidation of authenticity. As no clear correlation between the methoxypyrazine content and other wines parameters (alcohol and reducing sugar content, total acidity and pH) were observed, this also contributed no information regarding authenticity.

Unsuccessful classification of Sauvignon blanc wines may principally be attributed to the wide variation in the 3-alkyl-2-methoxypyrazine concentration of the samples, a phenomenon reported by various other authors.[5,7,11,12,17] The concentration of these components are reported to decline dramatically as ripening progress, more than 96% of the veraison level of IBMP was lost by normal harvesting maturity. Factors associated with this decline include ripening temperature, viticultural conditions (soil type, pruning and training, and plantation density) and light exposure .[7,10,11,12,17] Regarding light exposure, it was postulated that the berry concentration of IBMP is the result of two opposing effects, stimulation of biological formation on the one hand and photodegradation on the other.[17] It is also reported that IBMP mainly resides in stems, skins and seeds in Cabernet Sauvignon grapes, while relatively small amounts are located in the flesh. Although extractability was reported to play minor role, prolonged skin contact resulted in significant increases of IBMP content of juice.[19] Settling is reported to have an equally dramatic influence on the IBMP content of Sauvignon blanc must, clarification was reported to remove up to 50% of IBMP.[13,18] It may therefore be assumed that all the factors outlined above act together in a complex manner so that the final 3-alkyl-2-methoxypyrazine concentration of the samples are not simply the product of the vintage or region of origin but rather an intricate combination of diverse factors.

Finally it should be noted that neither of the two strategies that were used can provide absolute evidence of adulteration. As a very small amount of IBMP (1 to 2 ng/L) may have a significant effect on the aroma of Sauvignon blanc wine[6], manipulation of the aroma may be achieved by the addition of relatively small quantities of IBMP to wine. Since the variation in the IBMP content, as well as in the relative ratios, are relatively large in South African wines, the method may not be able to detect cases of adulteration where small, measured amounts of adulterants were added. Adulteration with small measured amounts may however not be easy to achieve (without sophisticated equipment) because of the very low quantities, and large volumes (of wine) involved. Synthetic preparations, where the methoxypyrazine content may possibly be certified, may offer unscrupulous producers an easy opportunity to manipulate the aroma of Sauvignon blanc wine undetected.

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CHAPTER 7

Summary and final concluding remarks

The South African Sauvignon blanc industry was under intense scrutiny for a number of years regarding adulteration of wines with foreign sources of 3-alkyl-2-methoxypyrazines.[1] In 2004, following an investigation conducted by the Wine and Spirit Board and the Agricultural Research Council (ARC Nietvoorbij), two producers admitted that illegal flavorants were added to their wines. The total volume of production of the relevant products were subsequently destroyed.[2] As adulteration constitutes a transgression of the Liquor Products Act[3], official samples of the contaminated products were confiscated by officials from the National Department of Agriculture while details of the case were passed on to the National Prosecuting Authority for consideration of possible legal action.

Regulation of the South African wine industry is a function that resorts under the National Department of Agriculture. As adulteration may tarnish the image of South African wines in the international market place and jeopardize the market share that South African producers command, it was decided that an extensive audit of the industry should be performed as a matter of the utmost urgency. Furthermore it was required that the two cases of adulteration be investigated and that evidence should be prepared to support possible litigation in this regard.

Within this context, the objectives of the study were two-fold in nature: Firstly a methodology for the determination of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wines was developed and implemented (Chapters 3, 4 and 5), and secondly a spectrum of wines representative of the industry were analyzed to establish parameters for authenticity elucidation and to perform an extensive audit of the industry (Chapter 6) at the same time. A comprehensive database of the 3-alkyl-2-methoxypyrazine content (Appendix 2, Table A) of South African Sauvignon blanc wines was thus established to provide information to enable the industry audit. Supplementary information yielded by possible sources of adulteration namely

green peppers and synthetic green pepper preparations, were also obtained in order to predict the effect of the addition thereof to wine. A small number of wines of other cultivars (Appendix 2, Table B) as well as Sauvignon blanc leaves were also analyzed.

Liquid chromatography-mass spectrometry (LC-MS) was selected as the analytical technique for measuring the levels of 3-alkyl-2-methoxypyrazines. The described method, utilizing distillation and solvent extraction for sample clean-up and pre-concentration followed by LC-MS determination of the residues, offered enhanced sensitivity compared to other techniques that were used for the same purpose. The method performance mostly allowed determination of the 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wines at their natural levels of occurrence. Table 7.1. summarize some of the performance characteristics of the method.

Table 7.1.: Performance characteristics of the method utilized for determination of 3-alkyl-2-methoxypyrazines in South African Sauvignon blanc wine.

Parameter	Value
MDL	0.03 ng/L
MQL	0.10 ng/L
Linear system response	Demonstrated for 5 to 4000 pg on column for IBMP and 5 to 1000 pg on column for IPMP and SBMP.
Recovery	Better than 90% (RSD < 10%) at levels 1, 10 and 100 ng/L
Instrument precision	RSD better than 5% utilizing aqueous standards.
Specificity	Molecular ion and one ion ratio for two secondary ion transitions were obtained.

The method described in this work represents the first known instance where LC-MS was applied for trace-level quantitation of 3-alkyl-2-methoxypyrazines in wine. The method produced improved levels of sensitivity compared to GC methods used for the same purpose and was successfully used to generate the most complete database pertaining to the 3-alkyl-2-methoxypyrazine content of South African Sauvignon blanc wine to date. The improved sensitivity realized with the method enabled the authors to quantify IBMP in all samples investigated as well as IPMP and SBMP in the majority of cases. The IBMP content was also measured in a

number of wines of other cultivars including for the first known instance in Pinotage, a unique South African cultivar. The improved sensitivity attained with the method therefore opens possibilities to study these components in wines which previously contained them in levels that were below the capabilities of known techniques.

A total of 577 South African Sauvignon blanc wines, from all wine-producing regions and of vintages 1999 to 2007, were analyzed. Due to sample availability constraints, only the 2004 and 2005 vintages were well represented with 197 and 295 samples respectively. Sauvignon blanc wines are mainly produced in the Breede River Valley and Coastal regions and as 118 and 268 samples were from these respective regions, sampling may be considered as reasonably representative regarding geographical origin of the wines.

Measured values for the IBMP concentration in this study ranged from 0.40 to 44 ng/L (RSD = 102%) with an average of 6.2 ng/L and median of 4.3 ng/L indicating that the distribution of values are not symmetrical, the majority of the samples were at the lower end of the scale. The concentrations of IPMP and SBMP in South African Sauvignon blanc wines were consistently lower than that of IBMP, and ranged from < 0.03 to 3.9 ng/L and < 0.03 to 3.2 ng/L respectively. The ratios of IPMP to IBMP, and SBMP to IBMP were determined to be approximately 5% in each case. The analysis of green peppers revealed that IBMP is similarly the dominant 3-alkyl-2-methoxypyrazine as was the case with the synthetic flavor preparations. However, the relative abundances of IPMP and SBMP were generally far smaller in these items compared to Sauvignon blanc wine. Two criteria were therefore identified that may be used for authenticity elucidation; the absolute concentration of IBMP, and the ratios of IPMP to IBMP and SBMP to IBMP.

The data was also analyzed to determine whether factors such as the methoxypyrazine content and other wine parameters (alcohol and reducing sugar content, total acidity and pH) may be correlated with the region of origin or vintage of the samples. Data analysis was performed using univariate statistics as well as PCA. The results revealed that only a superficial relationship between the 3-alkyl-2-methoxypyrazine content of the samples and some regions of origin could be established. No correlation with the vintage or any of the other wine parameters

could be established. Rigid parameters could therefore not be established for the elucidation of authenticity, the factors discussed above should be considered together to establish authenticity. The method was nevertheless able to discriminate the two adulterated samples based on disproportionately high levels of IBMP.

The two adulterated samples contained statistically significant elevated levels of IBMP while the ratios of abundance of IPMP and SBMP were consistent with adulteration. No additional cases of adulteration in the dataset were identified based on the said criteria, a finding that may serve to restore confidence in the wine industry as it would certainly appear that adulteration is not as widespread as it was at first assumed to be. In this regard it should be noted that the suspicion is that adulteration mainly took place in vintages earlier than 2004. Although a limited number of samples of these relatively early vintages were available, these revealed no indication of adulteration as discussed above. Where small, but significant (only 1 to 2 ng/L IBMP required to influence wine aroma) amounts of adulterants may have been added, the method could clearly not detect the duplicity. From a practical perspective, the addition of small amounts of methoxypyrazines to large volumes of wine to affect enrichment at levels that does not exceed the natural abundance, may be difficult to achieve without sophisticated equipment.

From the data it is also evident that it certainly is possible to produce Sauvignon blanc wine in South Africa that posses the desired and characteristic herbaceous aroma. A total of 82% of the samples contained IBMP above the perceptible level in white wine (2 ng/L) while 16% of the products contained more that 10 ng/L IBMP. Further research in the viticultural field may reveal the factors that contribute to the high levels of IBMP observed in some wines and possibly facilitate the production of wines with consistently high levels. Future method developments regarding detection of adulteration may possibly focus on other substances unique to green peppers and synthetic preparations (but not wine) that might be added to wine as a result of the adulteration process. However, such components if present at all would almost certainly be present at very low concentrations. The probability that a natural component of this nature, present at ultra-trace levels will be unique to green peppers and not to wine may be small. This method is therefore currently the only available option to determine adulteration of this nature and as it utilizes two sets of

parameters for authenticity elucidation, it represents an improvement on similar strategies utilized for the same purpose.

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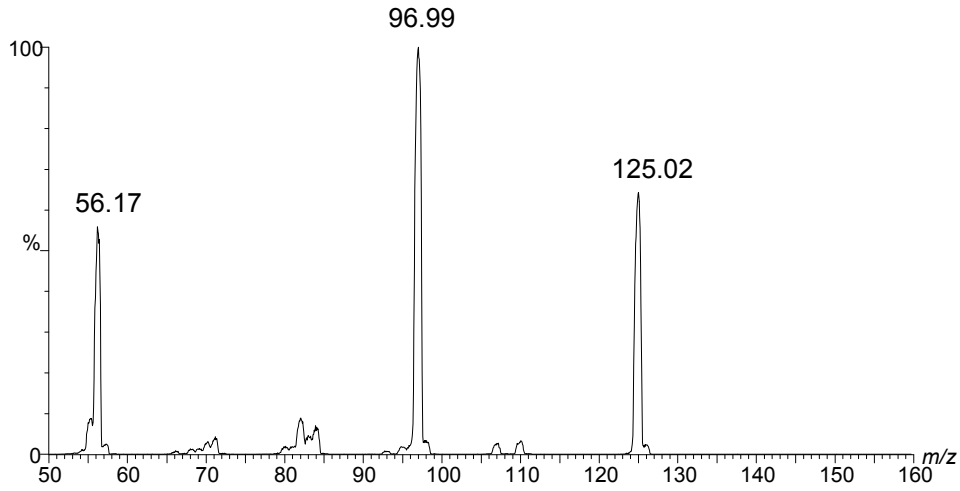
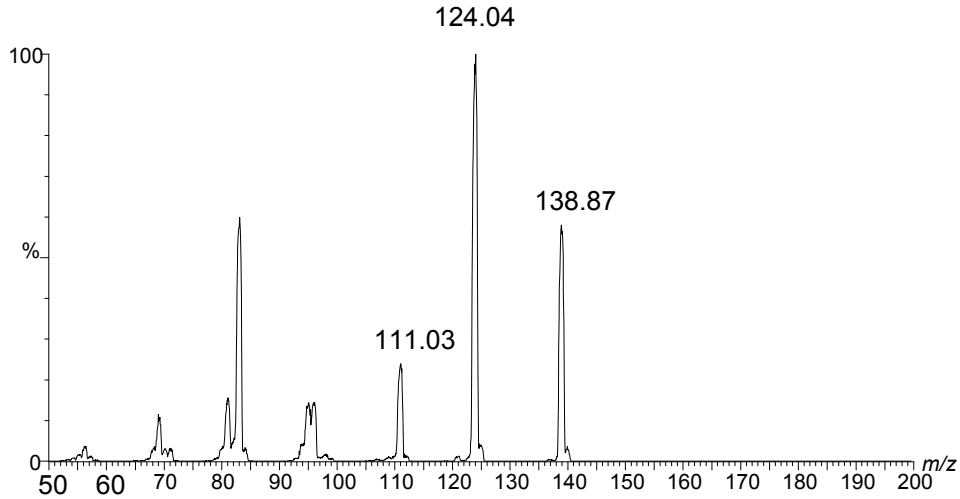
Appendix 1: Mass spectra of components of interest



3-Methyl-2-methoxypyrazine



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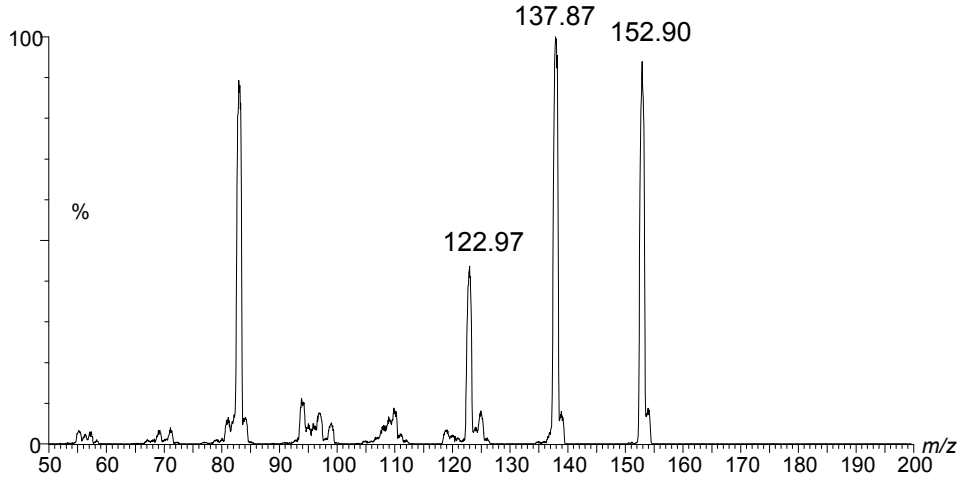
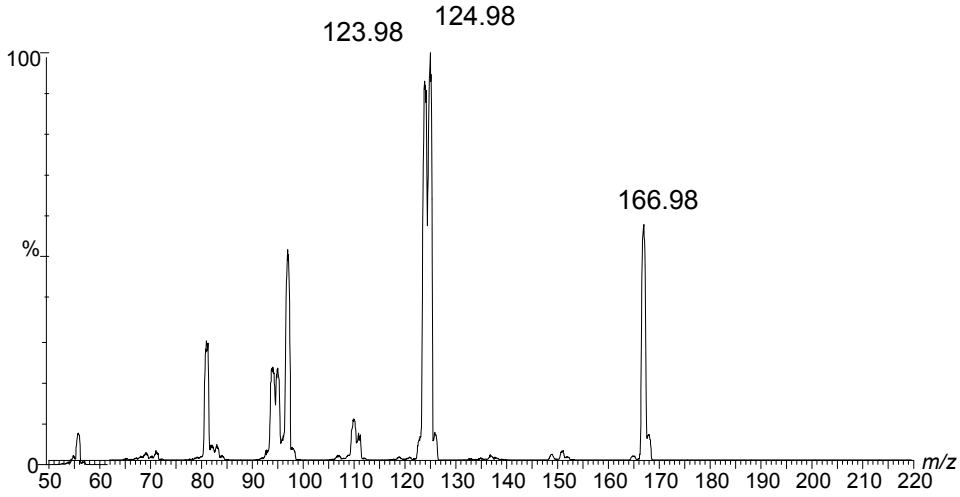
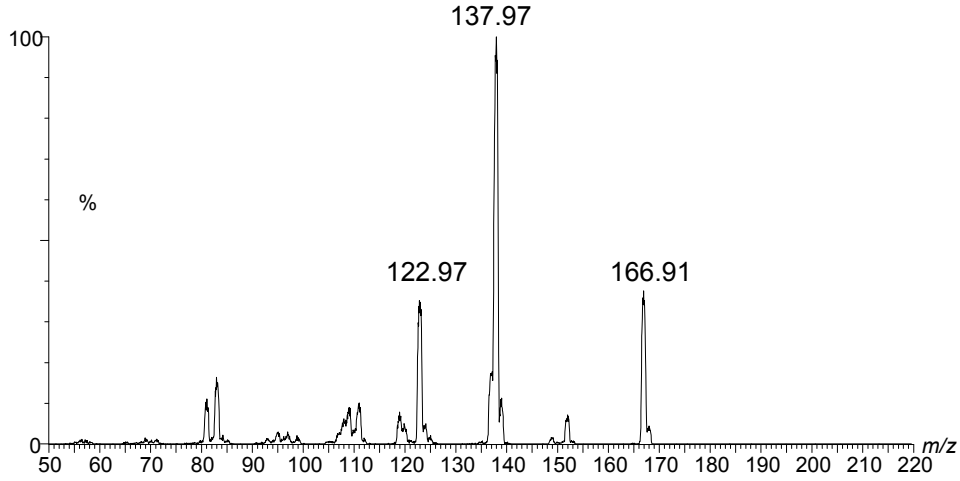


3-Isopropyl-2-methoxypyrazine



3-Isobutyl-2-methoxypyrazine



3-*sec*-butyl-2-methoxypyrazine

Appendix 2: Quantitative data for wines analyzed in the study

Table A: Data pertaining to Sauvignon blanc wine (main study).

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
1	Western Cape	2004	3.9	0.16	0.19	4.9	4.1	12.62	1.4	3.24	6.4
2	Western Cape	2004	4.8	0.22	0.15	3.1	4.6	12.23	1.6	3.58	6.0
3	Franschhoek	2004	0.82	NQ	ND	-	-	13.49	3.3	3.09	7.3
4	Franschhoek	2004	1.1	NQ	ND	-	-	13.41	1.3	3.05	7.1
5	Paarl	2004	4.6	0.18	0.16	3.5	3.9	15.10	1.3	3.49	4.9
6	Stellenbosch	2005	11	0.24	0.63	5.7	2.2	13.32	1.7	3.38	6.0
7	Stellenbosch	2002	2.5	NQ	0.13	5.2	-	13.32	2.5	3.19	6.0
8	Western Cape	2003	6.9	0.12	ND	-	1.7	12.71	1.5	3.36	5.8
9	Western Cape	2003	1.2	NQ	ND	-	-	13.41	1.5	3.35	7.3
10	Wellington	2004	6.9	0.25	0.67	9.7	3.6	12.28	2.5	3.19	6.3
11	Stellenbosch	2002	2.2	0.11	NQ	-	5.0	13.32	1.3	3.71	5.8
12	Western Cape	2004	3.2	0.12	NQ	-	3.8	12.62	4.2	3.28	6.8
13	Coastal region	2005	3.2	NQ	0.21	6.6	-	12.88	4.7	2.97	6.8
14	Coastal region	2005	3.4	0.12	0.11	3.2	3.5	12.88	4.7	2.97	6.8
15	Wellington	2005	1.6	ND	ND	-	-	11.76	5.3	3.36	7.0
16	Groenekloof	2005	15	0.47	1.3	8.4	3.1	12.71	1.3	3.39	7.3
17	Stellenbosch	2005	4.3	ND	NQ	-	-	13.49	1.7	3.07	7.1
18	Coastal region	2004	310	0.12	0.68	0.2	0.0				
19	n/o	2004	960	0.14	0.69	0.1	0.0				
20	Constantia	2004	18	0.40	1.1	6.1	2.2	12.97	1.6	3.38	6.9
21	Stellenbosch	2004	2.1	NQ	NQ	-	-	13.14	1.6	3.20	6.3
22	Coastal region	2004	8.7	0.30	0.64	7.4	3.4	13.06	1.3	3.17	6.6
23	Stellenbosch	2004	5.2	0.18	0.30	5.8	3.5	12.88	2.4	3.43	6.8
24	Western Cape	2003	1.6	NQ	NQ	-	-	13.36	1.7	3.19	6.5
25	Western Cape	2004	2.8	0.13	NQ	-	4.6	13.94	2.4	3.32	6.0
26	Constantia	2004	12	0.25	0.85	7.1	2.1	12.97	3.3	3.25	7.5
27	Western Cape	2004	4.7	0.16	0.63	13.4	3.4	13.37	2.7	3.80	4.8
28	Paarl	2003	3.1	0.10	0.14	4.5	3.2	12.88	1.4	3.44	4.9
29	Stellenbosch	2004	2.1	NQ	NQ	-	-	13.53	1.7	3.11	5.9
30	Swartland	2004	3.6	ND	0.11	3.1	-	12.19	1.6	3.48	5.9
31	Tulbagh	2004	2.2	0.12	ND	-	5.5	12.97	2.9	2.93	6.9
32	Coastal region	2005	18	0.29	1.0	5.6	1.6	13.23	1.8	3.47	6.9

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
33	Western Cape	2004	8.4	0.19	0.41	4.9	2.3	12.02	3.2	3.57	5.6
34	Robertson	2004	3.1	0.13	0.18	5.8	4.2	12.49	1.0	3.34	6.4
35	Robertson	2004	25	0.38	1.3	5.0	1.5	12.83	1.4	3.62	7.6
36	Robertson	2004	3.2	0.18	0.11	3.4	5.6	12.80	1.6	3.45	6.4
38	Robertson	2004	1.6	0.10	ND	-	6.3	13.76	3.1	3.36	6.7
39	Robertson	2004	1.7	0.10	ND	-	5.9	13.72	2.8	3.31	6.6
40	Worcester	2003	1.4	0.13	ND	-	9.3	13.21	7.5	3.43	6.5
41	Worcester	2002	2.2	0.15	ND	-	6.8	12.70	5.8	3.07	7.8
42	Slanghoek	2003	20	0.30	0.88	4.4	1.5	12.39	1.1	3.19	7.2
43	Breëriviervallei	2003	1.5	NQ	NQ	-	-	13.77	1.4	3.32	5.3
44	Goudini	2003	5.4	0.21	0.11	2.0	3.9	12.90	1.0	3.19	6.1
45	Robertson	2004	2.3	0.13	0.24	10.4	5.7	12.49	1.1	3.57	5.4
46	Robertson	2004	1.7	NQ	NQ	-	-	13.98	2.9	3.30	6.6
47	Robertson	2004	1.8	0.10	NQ	-	5.6	13.60	2.3	3.62	6.4
48	Worcester	2003	1.3	0.10	ND	-	7.7	13.19	2.3	3.42	6.8
49	Worcester	2003	2.5	0.15	0.17	6.8	6.0	12.25	1.2	3.46	5.8
50	Breëriviervallei	2003	0.72	NQ	NQ	-	-	13.19	1.4	3.16	5.8
51	Robertson	2004	0.66	NQ	NQ	-	-	13.01	1.3	3.20	5.9
52	Robertson	2004	3.2	0.20	0.17	5.3	6.3	11.93	1.3	3.39	7.5
53	Robertson	2004	24	0.31	1.3	5.5	1.3	12.45	1.3	3.58	8.0
54	Robertson	2004	5.7	0.17	0.17	3.0	3.0	12.38	2.0	3.49	8.0
56	Robertson	2003	1.9	0.13	NQ	-	6.8	12.01	1.4	3.60	6.4
57	Robertson	2004	3	0.12	NQ	-	4.0	12.10	1.2	3.11	6.2
58	Worcester	2002	1.8	0.13	NQ	-	7.2	12.93	2.7	3.41	6.3
59	Worcester	2003	2.7	0.26	0.11	4.1	9.6	13.41	7.2	3.11	7.0
60	Breëriviervallei	2002	1.7	0.13	0.12	7.1	7.6	12.63	2.6	3.46	6.4
61	Worcester	2003	3.1	0.36	0.10	3.2	11.6	13.07	9.4	3.18	7.1
62	Goudini	2003	5.8	0.24	0.33	5.7	4.1	12.94	3.6	3.18	6.2
63	Worcester	2002	16	0.46	0.19	1.2	2.9	12.78	1.2	3.53	5.0
64	Goudini	2003	3.6	0.13	NQ	-	3.6	11.48	1.0	3.50	6.9
65	Worcester	2003	6	0.22	0.23	3.8	3.7	11.59	1.1	3.57	6.8
66	Worcester	2003	11	0.11	0.23	2.1	1.0	11.41	1.1	3.41	7.0
67	Worcester	2003	2.8	0.29	NQ	-	10.4	13.33	7.8	3.16	6.6
68	Worcester	2003	3.2	0.18	0.10	3.1	5.6	11.10	1.0	3.59	7.0
69	Worcester	2003	2.3	0.10	NQ	-	4.3	10.79	1.3	3.28	6.3
70	Worcester	2004	5.1	0.29	0.25	4.9	5.7	12.67	1.6	3.34	6.5

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
71	Nuy	2004	5.9	0.23	0.36	6.1	3.9	12.97	1.3	3.36	7.0
72	Nuy	2004	4.5	0.19	0.22	4.9	4.2	13.14	1.8	3.29	6.6
73	Goudini	2004	11	0.28	0.62	5.6	2.5	13.14	5.1	3.33	7.4
74	Worcester	2004	3	0.17	0.28	9.3	5.7	12.19	1.4	3.25	6.4
75	Worcester	2004	4.3	0.12	NQ	-	2.8	12.31	3.3	3.20	5.8
76	Slanghoek	2004	11	0.31	0.96	8.7	2.8	11.81	4.2	3.37	7.6
77	Worcester	2004	6.1	0.21	0.23	3.8	3.4	12.10	1.0	3.46	6.1
78	Worcester	2004	2.8	0.19	0.21	7.5	6.8	12.19	3.0	3.29	6.3
80	Worcester	2004	4.6	0.39	0.22	4.8	8.5	12.40	3.9	3.61	7.3
81	Worcester	2004	5.4	0.16	0.17	3.1	3.0	12.62	2.0	3.49	6.5
82	Robertson	2004	3.9	0.14	NQ	-	3.6	11.34	1.4	3.41	6.4
83	Robertson	2004	25	0.44	1.1	4.4	1.8	12.31	1.5	3.53	7.4
84	Robertson	2004	1.2	NQ	NQ	-	-	13.36	2.8	3.13	6.9
86	Robertson	2004	11	0.26	0.24	2.2	2.4	13.30	1.3	3.39	5.7
87	Robertson	2004	3.9	0.10	0.15	3.8	2.6	12.16	4.6	3.31	7.1
88	Robertson	2004	4.6	0.11	0.44	9.6	2.4	11.88	1.4	3.40	6.7
89	Robertson	2004	3.8	0.18	0.21	5.5	4.7	12.05	1.0	3.07	5.8
90	Robertson	2004	2.7	0.16	NQ	-	5.9	13.46	2.7	3.30	7.8
91	Bonnievale	2004	4.6	0.18	0.48	10.4	3.9	12.49	3.5	3.25	6.9
92	Robertson	2004	2.8	0.18	0.13	4.6	6.4	12.45	1.4	3.33	6.5
93	Robertson	2004	11	0.30	0.60	5.5	2.7	11.62	2.0	3.19	6.5
94	Western Cape	2004	8.3	0.12	0.30	3.6	1.4	12.19	3.2	3.21	7.1
95	Western Cape	2004	4.2	0.15	0.22	5.2	3.6	12.71	1.3	3.33	6.9
96	Constantia	2005	20	3.2	2.6	12.9	15.8	13.58	2.6	3.25	6.3
97	Prince Albert Valley	2005	4.2	0.16	NQ	-	3.8	12.19	6.0	3.26	6.2
98	Robertson	2004	2.3	0.15	NQ	-	6.5	13.42	1.3	3.76	5.3
99	Robertson	2004	17	0.46	1.2	7.1	2.7	11.59	1.9	3.38	7.1
100	McGregor	2004	5.4	0.44	0.76	14.1	8.1	12.02	2.7	3.43	6.3
101	Robertson	2004	1.5	NQ	ND	-	-	12.10	1.4	3.21	6.2
103	Robertson	2004	5.3	0.21	0.27	5.1	4.0	12.56	1.5	3.52	7.6
104	Robertson	2004	2.7	0.18	0.23	8.5	6.7	12.80	1.5	3.44	6.4
105	McGregor	2004	4.1	0.36	0.40	9.8	8.8	12.02	3.2	3.30	6.8
106	Robertson	2004	2.8	0.20	0.13	4.6	7.1	12.80	1.6	3.45	6.4
107	Robertson	2004	5.1	0.22	0.45	8.8	4.3	12.28	3.0	3.52	6.5
108	Robertson	2004	1	0.13	NQ	-	13.0	13.49	2.8	3.25	6.7
109	McGregor	2004	1.7	0.24	0.15	8.8	14.1	12.31	3.4	3.33	6.2

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
110	Robertson	2004	24	0.45	1.6	6.8	1.9	12.66	1.3	3.56	7.7
111	Goudini	2004	4.4	0.18	0.27	6.1	4.1	12.31	1.1	3.33	6.4
112	Worcester	2004	5.8	0.23	0.29	5.0	4.0	12.14	1.0	3.44	5.7
113	Robertson	2004	1.4	0.13	NQ	-	9.3	12.14	1.1	3.50	5.7
114	Nuy	2003	2	0.16	NQ	-	8.0	12.46	1.6	3.21	6.4
115	Slanghoek	2003	17	0.22	0.22	1.3	1.3	12.36	3.0	3.28	7.3
116	Worcester	2003	2.5	0.15	NQ	-	6.0	12.50	1.2	3.45	5.8
117	Worcester	2003	2.3	0.15	0.19	8.3	6.5	13.15	3.2	3.32	6.0
118	Goudini	2003	4.1	0.22	0.22	5.4	5.4	12.51	2.4	3.36	5.8
119	Goudini	2003	4.9	0.18	0.26	5.3	3.7	12.78	1.7	3.40	5.6
120	Breëriviervallei	2003	1.3	0.10	NQ	-	7.7	13.83	2.5	3.43	6.8
121	Breëriviervallei	2003	1.3	0.13	0.10	7.7	10.0	13.49	1.2	3.39	7.0
122	Slanghoek	2004	10	0.31	0.91	9.1	3.1	11.76	1.2	3.45	6.7
123	Slanghoek	2004	26	0.78	3.3	12.8	3.0	13.98	1.5	3.27	6.0
124	Worcester	2004	6.3	0.37	0.54	8.6	5.9	11.53	1.4	3.38	6.3
125	Goudini	2004	9.3	0.24	0.52	5.6	2.6	11.10	2.9	3.71	5.7
126	Worcester	2004	3.1	0.23	0.24	7.7	7.4	11.79	1.5	3.39	5.9
127	Worcester	2004	17	0.56	0.73	4.3	3.3	11.79	1.2	3.44	6.7
128	Worcester	2004	3.1	0.14	0.17	5.5	4.5	13.25	1.2	3.36	6.3
129	Worcester	2004	4.7	0.27	0.32	6.8	5.7	12.75	3.8	3.59	5.8
130	Worcester	2004	4	0.25	0.22	5.5	6.3	12.75	3.3	3.48	5.9
131	Goudini	2004	5.7	0.15	NQ	-	2.6	11.09	1.3	3.51	6.4
132	Nuy	2004	5.6	0.26	0.25	4.5	4.6	12.49	2.7	3.32	6.6
133	Nuy	2004	5.9	0.27	0.21	3.6	4.6	12.66	1.1	3.22	7.8
134	Worcester	2003	2.8	0.33	0.11	3.9	11.8	13.08	2.0	3.28	6.1
135	Worcester	2003	1.9	0.30	0.12	6.3	15.8	13.36	5.0	3.28	6.4
137	Worcester	2003	9.4	0.20	0.19	2.0	2.1	13.15	2.6	3.41	6.2
138	Robertson	2004	2.3	0.17	0.17	7.4	7.4	11.09	1.1	3.40	7.1
139	Worcester	2003	2.6	0.17	0.31	11.9	6.5	13.03	3.8	3.28	6.8
140	Robertson	2004	1	0.15	NQ	-	15.0	12.28	2.8	3.36	6.4
141	Goudini	2003	2.5	0.22	NQ	-	8.8	13.26	1.5	3.39	6.5
142	Goudini	2003	2.7	0.19	0.11	4.1	7.0	13.27	1.0	3.51	5.8
143	Robertson	2004	2.7	0.21	0.12	4.4	7.8	12.88	3.3	3.48	5.7
145	Goudini	2003	4.4	0.30	0.18	4.1	6.8	12.18	1.3	3.28	7.4
146	Tradouw	2003	1.4	0.15	0.12	8.6	10.7	13.67	2.2	3.43	5.1
147	Coastal region	2003	18	0.28	0.20	1.1	1.6	12.62	2.7	3.44	5.9

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
148	Coastal region	2003	4.5	0.23	NQ	-	5.1	12.80	3.3	3.34	6.6
149	Durbanville	2005	6.3	0.23	0.32	5.1	3.7	12.28	2.7	3.41	5.5
150	Durbanville	2005	11	0.23	0.33	3.0	2.1	13.27	1.6	3.53	6.3
151	Western Cape	2005	3.4	0.20	0.36	10.6	5.9	13.15	2.6	3.44	6.9
152	Stellenbosch	2005	2	0.15	NQ	-	7.5	13.33	1.1	3.47	6.0
153	Simonsberg	2005	5.9	0.24	0.34	5.8	4.1	12.80	1.7	3.36	7.6
154	Coastal region	2005	9.2	0.27	0.48	5.2	2.9	12.71	1.9	3.50	6.5
155	Durbanville	2003	4.2	0.26	0.39	9.3	6.2	11.85	1.6	3.40	5.8
156	Stellenbosch	2005	3.6	0.20	NQ	-	5.6	14.14	3.1	3.50	6.0
157	Stellenbosch	2005	5.1	0.17	0.14	2.7	3.3	13.46	1.9	3.32	6.9
158	Bottelary	2005	2.5	0.14	NQ	-	5.6	13.76	2.4	3.26	6.2
159	Western Cape	2002	2.6	0.18	0.21	8.1	6.9	12.57	8.6	3.15	6.8
160	Western Cape	2005	1	0.13	NQ	-	13.0	11.76	1.3	3.23	6.6
161	Western Cape	2005	4.9	0.21	0.15	3.1	4.3	12.36	2.4	3.30	6.1
162	Stellenbosch	2005	5	0.23	0.18	3.6	4.6	12.28	1.2	3.49	6.7
164	Franschhoek	2005	1.1	0.11	NQ	-	10.0	14.85	1.3	3.32	5.9
165	Constantia	2005	9.6	0.71	0.75	7.8	7.4	13.32	1.7	3.40	7.0
166	Western Cape	2005	27	0.57	2.4	9.0	2.1	14.20	5.0	3.45	6.3
167	Paarl	2002	17	0.30	0.50	2.9	1.8	13.56	6.5	3.27	5.2
168	Lutzville	2005	4.6	0.19	0.14	3.0	4.1	11.85	1.0	3.27	6.1
169	Tulbagh	2004	2	0.10	NQ	-	5.0	12.97	2.8	3.12	6.6
170	Western Cape	2005	12	0.49	1.5	12.8	4.1	12.19	1.3	3.56	5.8
171	Elim	2005	9.5	0.24	0.27	2.8	2.5	13.52	1.5	3.44	6.8
172	Durbanville	2004	3.6	0.19	0.35	9.7	5.3	13.32	2.4	3.16	5.9
173	Western Cape	2005	2.8	NQ	NQ	-	-	12.78	3.7	3.16	6.3
174	Western Cape	2005	3	0.22	0.10	3.3	7.3	12.93	4.8	3.42	6.3
175	Western Cape	2005	1.5	0.10	ND	-	6.7	13.02	1.9	3.13	7.7
176	Stellenbosch	2005	4.4	0.17	0.17	3.9	3.9	13.43	1.3	3.35	7.0
177	Coastal region	2005	6.3	0.25	0.20	3.2	4.0	12.62	2.6	3.37	7.6
178	Western Cape	2005	2.3	0.17	0.49	21.3	7.4	11.85	2.7	3.55	5.9
179	Durbanville	2005	7.2	0.23	0.50	6.9	3.2	14.29	1.8	3.49	6.0
180	Coastal region	2005	2.5	0.15	0.12	4.8	6.0	13.23	3.9	3.35	7.4
181	Walker Bay	2005	2	0.12	0.10	5.0	6.0	12.59	3.4	3.11	7.4
182	Western Cape	2005	1.6	0.12	NQ	-	7.5	13.92	2.5	3.42	6.1
183	Stellenbosch	2005	5.8	0.25	0.34	5.9	4.3	12.41	1.9	3.33	7.1
184	Coastal region	2004	8.7	0.30	0.64	7.4	3.4	12.64	1.7	3.40	5.7

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
185	Stellenbosch	2003	5.7	0.23	0.56	9.8	4.0	13.35	1.5	3.47	7.1
186	Coastal region	2005	2.3	0.12	0.10	4.3	5.2	13.22	2.3	3.24	6.7
187	Coastal region	2004	5	0.37	0.15	3.0	7.4	11.68	2.5	3.40	6.3
188	Western Cape	2004	4	0.28	0.48	12.0	7.0	12.53	2.0	3.15	6.5
189	Western Cape	2005	1.2	0.14	NQ	-	11.7	12.98	1.1	3.29	5.5
190	Paarl	2004	4.4	0.16	0.29	6.6	3.6	13.30	3.7	3.31	6.6
191	Western Cape	2005	2.3	0.17	NQ	-	7.4	11.58	3.5	3.24	7.2
192	Western Cape	2005	4.8	0.30	0.32	6.7	6.3	12.11	2.6	3.42	5.9
193	Stellenbosch	2005	1.2	0.13	0.10	8.3	10.8	11.85	4.4	3.04	7.0
194	Coastal region	2004	5.8	0.24	0.35	6.0	4.1	13.67	2.5	3.26	7.1
195	Coastal region	2003	7.6	0.54	0.58	7.6	7.1	13.38	2.0	3.25	7.9
196	Durbanville	2003	4	0.20	0.36	9.0	5.0	12.23	1.6	3.41	5.9
197	Stellenbosch	2004	0.88	NQ	NQ	-	-	15.07	2.1	3.17	6.9
198	Stellenbosch	2005	7.2	0.22	0.28	3.9	3.1	13.06	2.7	3.45	5.7
199	Stellenbosch	2005	6.5	0.12	0.23	3.5	1.8	13.59	2.7	3.53	6.3
200	Franschhoek	2005	5.3	0.12	NQ	-	2.3	12.20	2.7	3.38	7.9
201	Stellenbosch	2005	3.8	0.14	NQ	-	3.7	13.39	3.8	3.28	6.6
202	Elgin	2005	10	0.34	0.45	4.5	3.4	12.28	1.0	3.28	6.9
203	Western Cape	2005	7.9	0.26	0.70	8.9	3.3	13.03	3.5	3.14	7.9
204	Darling	2005	13	0.29	0.79	6.1	2.2	14.04	2.2	3.60	6.6
205	Paarl	2005	1.6	0.16	ND	-	10.0	12.87	3.7	3.21	6.4
206	Wellington	2005	0.66	0.13	ND	-	19.7	12.02	1.4	3.13	6.5
207	Paarl	2005	0.52	0.32	ND	-	61.5	12.48	1.5	3.66	5.1
208	Paarl	2004	0.84	0.12	NQ	-	14.3	13.29	2.0	3.15	6.5
209	Stellenbosch	2005	3.8	0.18	NQ	-	4.7	13.14	2.4	3.32	6.4
210	Western Cape	2005	6.5	0.20	0.21	3.2	3.1	12.61	2.4	3.48	6.7
211	Stellenbosch	2005	16	0.50	1.2	7.4	3.1	13.23	2.5	3.24	6.4
212	Stellenbosch	2005	1.1	NQ	NQ	-	-	13.58	2.5	3.14	6.7
213	Western Cape	2005	3.3	0.16	NQ	-	4.8	12.95	3.6	3.27	6.4
214	Stellenbosch	2003	3.3	0.18	0.21	6.4	5.5	13.56	2.2	3.20	7.6
215	Walker Bay	2005	4.9	0.20	0.15	3.1	4.1	13.51	4.4	3.19	7.0
216	Western Cape	2005	2.8	0.20	0.28	10.0	7.1	12.45	2.4	3.43	5.9
218	Walker Bay	2005	2.6	0.10	NQ	-	3.8	13.32	1.2	3.25	6.6
219	Constantia	2005	14	1.4	1.5	10.6	9.7	13.67	1.5	3.42	6.9
221	Western Cape	2005	2	0.12	NQ	-	6.0	12.57	5.9	3.39	5.6
222	Wellington	2005	1.8	0.11	ND	-	6.1	12.19	1.5	3.15	7.2

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
223	Coastal region	2004	5.3	0.20	0.15	2.8	3.8	12.01	5.3	3.36	7.0
224	Coastal region	2005	3.9	0.12	0.21	5.4	3.1	12.27	2.4	3.26	6.0
225	Coastal region	2005	4	0.16	0.20	5.0	4.0	12.02	2.3	3.22	6.3
226	Cederberg	2005	5.9	0.30	0.19	3.2	5.1	13.29	2.1	3.25	8.3
228	Walker Bay	2004	1.4	0.13	0.10	7.1	9.3	12.88	2.6	3.16	6.7
229	Coastal region	2003	3.2	0.16	0.16	5.0	5.0	12.97	3.5	3.37	6.2
230	Western Cape	2005	3.3	0.18	0.13	3.9	5.5	11.26	4.1	3.44	5.9
232	Western Cape	2004	5.8	0.17	0.12	2.1	2.9	11.17	7.3	3.53	5.8
233	Stellenbosch	2004	2.3	0.12	0.17	7.4	5.2	12.71	1.2	3.34	6.0
234	Coastal region	2004	12	0.26	0.51	4.3	2.2	12.45	1.6	3.45	5.8
235	Western Cape	2004	5.8	0.25	0.35	6.0	4.3	11.59	2.7	3.42	6.3
236	Western Cape	2004	5.9	0.21	0.17	2.9	3.6	12.19	3.4	3.40	6.0
237	Western Cape	2003	4.1	0.16	0.20	4.9	3.9	12.80	2.9	3.41	5.6
238	Klein Rivier	2004	26	0.77	2.5	9.7	3.0	12.62	1.3	3.62	5.5
239	Stellenbosch	2004	1.2	0.11	NQ	-	9.2	13.23	1.4	3.32	6.5
240	Western Cape	2004	4.1	0.12	0.21	5.1	2.9	12.88	1.4	3.38	6.0
241	Darling	2004	9.1	0.31	0.51	5.6	3.4	13.06	2.4	3.45	6.6
242	Coastal region	2004	12	0.33	0.72	6.0	2.8	12.53	1.7	3.46	5.5
243	Devon Valley	2003	5.1	0.15	0.18	3.5	2.9	13.40	2.4	3.24	7.3
244	Western Cape	2004	5.5	0.20	0.13	2.4	3.6	12.02	2.8	3.55	6.0
245	Western Cape	2006	5.1	0.27	0.19	3.7	5.3	12.28	3.5	3.39	6.1
246	Coastal region	2005	7.7	0.19	0.32	4.2	2.5	12.97	2.6	3.16	6.7
247	Constantia	2005	6.7	0.24	0.26	3.9	3.6	13.06	3.2	3.09	6.8
248	Stellenbosch	2004	3.5	0.14	0.11	3.1	4.0	12.97	3.5	3.37	6.0
249	Coastal region	2003	0.4	0.11	ND	2.5	27.5	13.32	4.5	3.51	6.4
250	Stellenbosch	2004	4.3	0.13	0.10	2.3	3.0	12.02	2.5	3.36	6.5
251	Stellenbosch	2004	6.1	0.25	0.24	3.9	4.1	11.93	1.5	3.27	7.5
252	Durbanville	2004	13	0.25	0.95	7.3	1.9	12.57	2.3	3.47	6.6
253	Stellenbosch	2004	4.4	0.20	0.27	6.1	4.5	12.45	5.4	3.18	7.4
254	Stellenbosch	2004	3.6	0.15	0.13	3.6	4.2	12.53	3.3	3.33	7.4
255	Franschhoek	2004	1.8	0.11	NQ	-	6.1	13.41	2.2	3.19	7.0
256	Coastal region	2004	11	0.34	0.70	6.4	3.1	12.45	2.1	3.19	6.3
257	Western Cape	2004	5.5	0.18	0.37	6.7	3.3	12.88	4.1	3.25	6.0
258	Elgin	2004	2.3	0.11	NQ	-	4.8	12.71	1.5	3.10	7.3
259	Western Cape	2004	8.4	0.35	0.81	9.6	4.2	12.19	2.2	3.47	6.0
260	Groenekloof	2005	16	0.50	1.1	6.8	3.1	12.80	1.5	3.51	7.1

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
261	Robertson	2006	4	0.27	0.18	4.5	6.8	11.26	3.9	3.18	7.1
262	Elim	2006	34	0.88	2.0	5.9	2.6	13.85	5.5	3.31	6.2
263	Darling	2006	8.2	0.25	0.59	7.2	3.0	13.41	1.2	3.39	5.9
264	Western Cape	2005	4.8	0.16	0.26	5.4	3.3	12.36	3.8	3.25	6.5
265	Swartland	2006	2.9	0.17	0.18	6.2	5.9	12.45	1.3	3.25	6.0
266	Coastal region	2004	9.8	0.35	0.68	6.9	3.6	12.62	2.7	3.40	5.6
267	Western Cape	2005	3.3	0.16	0.10	3.0	4.8	13.14	3.1	3.13	6.9
268	Western Cape	2005	8.7	NQ	NQ	-	-	12.36	4.6	3.50	6.4
269	Western Cape	2005	1.6	0.12	ND	-	7.5	12.36	5.5	3.46	5.9
270	Western Cape	2005	1.7	0.14	NQ	-	8.2	11.85	6.2	3.30	7.2
271	Coastal region	2006	4.1	0.29	0.39	9.5	7.1	12.71	1.7	3.23	6.8
272	Coastal region	2006	9.8	0.28	0.52	5.3	2.9	12.45	3.5	3.25	7.3
273	Groenekloof	2004	4.6	0.19	0.45	9.8	4.1	13.32	1.9	2.96	7.3
274	Groenekloof	2006	5.9	0.21	0.30	5.1	3.6	13.58	1.6	3.28	6.8
275	Western Cape	2005	5	0.20	0.45	9.0	4.0	12.62	4.0	3.27	6.4
276	Stellenbosch	2004	2.5	0.10	0.16	6.4	4.0	14.04	3.8	3.30	6.9
277	Simonsberg	2005	4.5	0.16	0.18	4.0	3.6	13.32	2.0	3.21	6.6
279	Paarl	2006	2.1	0.24	NQ	-	11.4	11.59	1.1	3.23	5.5
280	Coastal region	2005	9.7	0.26	0.29	3.0	2.7	11.53	1.6	3.31	4.9
281	Paarl	2005	1.4	0.11	NQ	2.9	7.9	13.23	2.4	3.08	6.0
282	Walker Bay	2005	2.3	0.10	NQ	-	4.3	13.23	2.0	3.23	5.7
283	Stellenbosch	2004	3.2	0.16	0.13	4.1	5.0	12.62	2.6	3.34	6.2
284	Coastal region	2004	5.1	0.20	0.20	3.9	3.9	12.80	1.6	3.23	6.3
285	Western Cape	2004	1.7	0.13	NQ	-	7.6	13.49	1.9	3.34	6.8
286	Coastal region	2006	17	0.42	0.94	5.5	2.5	13.06	3.3	3.24	6.8
287	Western Cape	2004	4.9	0.29	0.23	4.7	5.9	11.17	3.9	3.39	6.4
288	Western Cape	2006	9.4	0.38	0.70	7.4	4.0	12.83	3.1	3.08	7.4
289	Coastal region	2005	10	0.22	0.31	3.1	2.2	12.60	2.2	3.44	6.3
290	Paarl	2005	1.1	0.17	NQ	-	15.5	12.40	3.0	3.58	5.7
291	Paarl	2005	24	0.23	0.11	0.5	1.0	13.49	2.3	3.44	6.6
292	Stellenbosch	2006	3.3	0.15	NQ	-	4.5	13.37	1.8	3.22	6.6
293	Stellenbosch	2005	5.3	0.20	0.14	2.6	3.8	12.48	3.1	3.29	6.4
294	Walker Bay	2006	25	1.3	1.6	6.6	5.2	12.85	1.8	3.14	7.3
295	Western Cape	2006	23	0.63	1.9	8.1	2.7	12.95	3.6	3.25	6.5
296	Lutzville	2006	31	0.74	2.2	7.2	2.4	12.56	2.8	3.18	8.3
297	Elgin	2004	2.8	0.18	0.11	3.9	6.4	12.57	1.6	2.99	7.1

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
298	Franschhoek	2004	2.1	0.13	0.12	5.7	6.2	12.40	3.1	3.20	5.9
299	Simonsberg	2004	3.3	0.20	0.28	8.5	6.1	12.30	1.4	3.42	6.8
301	Paarl	2004	7.8	0.10	0.12	1.5	1.3	13.56	2.2	3.43	6.5
303	Simonsberg	2004	0.76	0.10	NQ	-	13.2	12.92	1.6	2.92	7.7
304	Coastal region	2006	7.1	0.36	0.26	3.7	5.1	13.81	3.3	3.22	6.5
305	Western Cape	2005	2.1	0.11	NQ	-	5.2	12.38	5.8	3.37	6.8
306	Elgin	2005	5.5	0.22	0.23	4.2	4.0	13.32	2.5	3.25	6.8
308	Robertson	2004	2.8	0.18	0.15	5.4	6.4	12.75	1.7	3.52	5.9
309	Robertson	2004	26	0.39	1.7	6.6	1.5	12.45	1.5	3.57	7.5
310	Robertson	2004	4.6	0.26	0.29	6.3	5.7	12.71	3.1	3.37	6.0
311	Robertson	2004	2.9	0.20	0.11	3.8	6.9	12.88	2.9	3.43	5.8
312	Robertson	2004	3.5	0.19	0.11	3.1	5.4	11.76	1.1	3.14	6.3
313	Robertson	2004	4.2	0.15	NQ	-	3.6	11.43	1.3	3.35	6.8
314	Robertson	2004	4.1	0.16	0.81	19.8	3.9	12.62	3.1	3.44	7.1
315	Robertson	2004	25	0.47	1.6	6.6	1.9	12.88	1.5	3.60	7.5
316	Robertson	2004	2.3	0.11	0.11	4.8	4.8	13.58	2.6	3.27	6.4
317	Robertson	2004	1.1	NQ	NQ	-	-	13.76	3.5	3.15	6.8
318	Robertson	2004	1.3	NQ	ND	-	-	13.58	3.3	3.34	6.8
319	Elim	2006	42	0.87	2.2	5.1	2.1	13.54	6.2	2.84	6.4
320	Coastal region	2004	7.6	0.25	0.91	12.0	3.3	12.44	2.3	3.19	6.4
321	Paarl	2004	7.5	0.14	0.17	2.3	1.9	13.39	2.2	3.46	6.6
322	Coastal region	2004	5.3	0.26	0.26	4.9	4.9	13.47	2.0	3.14	6.8
323	Western Cape	2004	4.1	0.16	0.32	7.8	3.9	12.59	2.1	3.25	5.6
324	Western Cape	2006	0.58	0.13	NQ	-	22.4	12.97	2.2	3.12	7.8
325	Western Cape	2006	3.5	0.19	0.19	5.4	5.4	12.19	4.6	3.24	6.5
326	Stellenbosch	2005	3.2	0.19	0.10	3.1	5.9	13.94	3.0	3.40	6.2
327	Western Cape	2006	5.6	0.30	0.32	5.7	5.4	13.14	2.0	3.13	6.5
328	Robertson	2006	13	0.47	1.1	8.1	3.6	12.97	1.4	3.18	6.3
329	Coastal region	2006	14	0.37	0.66	4.7	2.6	12.88	3.1	3.32	7.2
330	Stellenbosch	2004	3	0.14	0.15	5.0	4.7	13.32	2.2	3.22	6.8
331	Constantia	2004	14	0.28	0.78	5.6	2.0	12.88	3.1	3.14	7.2
332	Paarl	2004	2.5	0.22	NQ	-	8.8	12.97	1.4	3.71	5.6
333	Stellenbosch	2003	3.3	0.26	0.20	6.1	7.9	12.97	1.4	3.08	6.7
334	Coastal region	2006	7.1	0.27	0.42	5.9	3.8	13.85	2.9	3.17	6.5
335	Walker Bay	2005	2.4	0.13	NQ	-	5.4	13.41	2.5	3.26	5.8
336	Western Cape	2005	4.2	0.15	NQ	-	3.6	13.94	1.9	3.21	6.9

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
337	Western Cape	2005	2.5	0.18	NQ	-	7.2	13.14	1.3	3.03	6.5
338	Western Cape	2005	1.8	0.13	0.11	6.1	7.2	12.62	2.6	3.51	6.0
339	Western Cape	2005	1.9	0.17	NQ	-	8.9	11.76	2.2	3.29	6.5
340	Western Cape	2005	1.8	0.14	NQ	-	7.8	13.41	4.1	3.19	6.6
341	Western Cape	2005	1.2	0.14	NQ	-	11.7	13.23	1.4	3.22	6.0
342	Western Cape	2005	4	0.21	0.16	4.0	5.3	12.62	1.7	3.36	6.2
343	Stellenbosch	2005	6.9	0.22	0.19	2.8	3.2	12.88	2.7	3.27	5.6
344	Western Cape	2005	2	0.15	NQ	-	7.5	12.88	3.2	3.50	5.5
345	Stellenbosch	2005	4.3	0.16	0.16	3.7	3.7	13.58	2.8	3.29	6.0
346	Western Cape	2005	2.6	0.19	0.19	7.3	7.3	11.76	1.8	3.29	6.8
347	Western Cape	2005	3.6	0.20	0.10	2.8	5.6	12.28	3.2	3.57	5.7
348	Stellenbosch	2005	6.8	0.14	0.37	5.4	2.1	12.36	4.7	3.23	7.9
349	Western Cape	2005	2.2	0.17	NQ	-	7.7	11.56	3.4	3.26	6.4
350	Stellenbosch	2004	7.2	0.16	0.11	1.5	2.2	11.68	3.2	3.44	5.5
351	Western Cape	2005	2.9	0.13	0.22	7.6	4.5	11.93	2.4	3.47	6.1
352	Stellenbosch	2005	4.9	0.17	0.24	4.9	3.5	13.58	2.5	3.40	6.4
353	Western Cape	2004	9.1	0.23	0.30	3.3	2.5	12.17	2.0	3.55	5.9
354	Swartland	2005	3.9	0.20	NQ	-	5.1	13.49	1.6	3.61	6.4
355	Durbanville	2005	8.5	0.19	0.39	4.6	2.2	12.80	1.9	3.43	6.7
356	Western Cape	2005	5.1	0.10	0.17	3.3	2.0	13.41	2.1	3.68	5.8
357	Coastal region	2005	1.3	0.11	NQ	-	8.5	12.97	1.3	3.50	5.8
358	Western Cape	2005	4.5	0.13	0.24	5.3	2.9	13.06	2.4	3.19	6.9
359	Western Cape	2005	3.1	0.76	0.36	11.6	24.5	11.68	2.4	3.45	6.8
360	Elim	2005	14	0.48	1.2	8.5	3.4	13.06	2.0	3.26	6.5
361	Coastal region	2005	3.1	0.17	NQ	-	5.5	12.02	1.8	3.21	6.7
362	Western Cape	2005	5.9	0.20	0.41	6.9	3.4	11.68	4.6	3.17	7.0
363	Western Cape	2005	3.1	0.20	ND	-	6.5	12.45	2.2	3.27	7.2
364	Western Cape	2005	1.1	0.14	NQ	-	12.7	12.97	1.7	3.31	7.4
365	Western Cape	2005	2.6	0.19	ND	-	7.3	11.68	2.2	3.36	6.6
366	Western Cape	2005	1.9	0.14	NQ	-	7.4	13.85	5.3	3.45	6.5
367	Western Cape	2005	2.3	0.15	NQ	-	6.5	12.19	3.1	3.26	6.1
368	Western Cape	2005	1.3	0.18	ND	-	13.8	13.17	3.2	3.41	7.3
369	Western Cape	2005	1.1	0.17	NQ	-	15.5	12.28	6.3	3.24	6.8
370	Western Cape	2005	2.6	0.15	0.13	5.0	5.8	12.28	2.3	3.44	6.6
371	Stellenbosch	2005	4.6	0.37	0.22	4.8	8.0	12.28	2.3	3.41	6.2
372	Western Cape	2005	1.9	0.15	NQ	-	7.9	12.71	3.2	3.50	6.7

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
373	Wellington	2005	1.4	0.18	NQ	-	12.9	12.10	2.9	3.30	6.1
374	Paarl	2005	6	0.23	0.21	3.5	3.8	13.58	1.6	3.32	7.4
375	Western Cape	2005	1.1	0.16	NQ	-	14.5	12.88	1.3	3.22	5.2
376	Western Cape	2005	1.6	0.16	ND	-	10.0	12.36	1.3	3.44	5.3
377	Paarl	2004	4.4	0.24	0.29	6.6	5.5	13.49	2.2	3.22	6.3
378	Western Cape	2005	3.9	0.21	NQ	-	5.4	11.76	3.7	3.26	6.2
379	Western Cape	2005	2.3	0.17	NQ	-	7.4	12.02	4.5	3.22	6.2
380	Western Cape	2005	0.98	0.16	ND	-	16.3	12.10	4.0	3.44	6.3
381	Western Cape	2005	0.94	0.31	NQ	-	33.0	12.36	1.2	3.25	6.5
382	Western Cape	2005	1.8	0.22	NQ	-	12.2	12.36	1.4	3.11	6.5
383	Stellenbosch	2004	3.1	0.21	0.18	5.8	6.8	12.97	2.5	3.26	6.4
384	Western Cape	2005	2.2	0.16	NQ	-	7.3	14.47	4.8	3.64	5.1
385	Western Cape	2005	2	0.18	ND	-	9.0	11.85	4.4	3.17	6.2
386	Western Cape	2005	1.8	0.15	ND	-	8.3	11.51	4.2	3.31	7.0
387	Western Cape	2005	4.4	0.19	0.18	4.1	4.3	12.88	2.0	3.48	6.8
388	Western Cape	2005	3.4	0.14	NQ	-	4.1	12.71	6.2	3.40	7.4
389	Western Cape	2005	6.1	0.18	0.10	1.6	3.0	12.02	4.0	3.28	7.1
391	Coastal region	2004	5.3	0.22	0.35	6.6	4.2	12.62	2.3	3.33	5.9
393	Coastal region	2003	22	0.27	0.20	0.9	1.2	13.23	1.9	3.35	5.9
394	Western Cape	2005	2.8	0.23	0.19	6.8	8.2	11.85	1.2	3.43	6.5
395	Western Cape	2004	8.6	0.27	0.37	4.3	3.1	12.45	1.6	3.23	6.0
396	Stellenbosch	2004	8.8	0.30	0.46	5.2	3.4	13.88	4.5	3.36	6.3
397	Western Cape	2004	2.8	0.18	0.28	10.0	6.4	12.33	3.3	3.50	5.8
398	Western Cape	2004	4.4	0.20	0.23	5.2	4.5	12.28	6.9	3.46	6.2
399	Paarl	2004	5.7	0.19	0.21	3.7	3.3	13.15	1.2	3.57	7.2
400	Cape Point	2004	13	0.61	0.70	5.4	4.7	13.23	2.5	3.29	6.5
401	Paarl	2004	1.7	0.18	0.13	7.6	10.6	13.24	2.7	3.04	5.8
402	Bottelary	2004	2.3	0.18	0.17	7.4	7.8	13.94	5.6	3.01	7.8
403	Paarl	2004	2.1	0.19	0.12	5.7	9.0	13.14	3.0	2.97	8.9
404	Western Cape	2004	4.7	0.22	0.30	6.4	4.7	11.09	3.6	3.46	5.8
405	Stellenbosch	2004	8.6	0.37	0.78	9.1	4.3	14.29	1.4	3.42	5.6
406	Robertson	2006	4.4	0.17	0.13	3.0	3.9	13.49	3.4	3.20	8.4
407	Stellenbosch	2006	2.8	0.18	ND	-	6.4	13.58	3.3	3.42	5.8
408	Coastal region	2005	8.2	0.25	0.40	4.9	3.0	12.88	1.5	3.33	6.4
410	Darling	2005	19	0.61	1.4	7.4	3.2	12.97	1.5	3.40	7.4
411	Simonsberg	2005	3.9	0.44	0.14	3.6	11.3	12.02	1.4	3.26	6.8

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
412	Paarl	2005	13	0.24	0.33	2.5	1.8	13.06	2.6	3.52	6.6
413	Elgin	2005	9.1	0.32	0.54	5.9	3.5	13.49	1.6	3.24	6.6
414	Constantia	2005	6.5	0.32	0.36	5.5	4.9	12.73	2.8	3.26	6.6
415	Stellenbosch	2005	1	0.11	ND	-	11.0	13.41	3.3	3.43	6.0
416	Simonsberg	2005	5.1	0.20	0.12	2.4	3.9	13.32	2.0	3.21	6.6
417	Durbanville	2004	9.6	0.31	0.58	6.0	3.2	12.71	1.5	3.05	7.0
418	Franschhoek	2005	0.9	0.11	NQ	-	12.2	12.71	2.0	3.29	6.8
419	Western Cape	2005	4.1	0.20	0.18	4.4	4.9	12.53	2.9	3.26	6.5
420	Western Cape	2005	2.8	0.21	ND	-	7.5	11.26	2.8	3.19	6.5
421	Western Cape	2005	2.1	0.17	0.34	16.2	8.1	12.45	2.4	3.43	5.9
422	Coastal region	2005	4.2	0.24	0.21	5.0	5.7	14.11	4.1	3.29	6.6
423	Franschhoek	2005	1.1	NQ	ND	-	-	12.71	3.0	2.98	7.5
424	Western Cape	2005	2.3	0.19	0.12	5.2	8.3	13.06	1.4	3.45	5.9
425	Cape Agulhas	2005	4.2	0.18	0.23	5.5	4.3	12.62	2.2	3.36	5.8
426	Cape Agulhas	2005	9.6	0.23	0.20	2.1	2.4	14.56	2.4	3.15	6.9
427	Coastal region	2005	3.1	0.18	0.20	6.5	5.8	13.06	4.0	3.04	7.3
428	Coastal region	2005	5.8	0.33	0.39	6.7	5.7	13.14	4.3	3.10	7.0
429	Western Cape	2005	0.72	0.13	ND	-	18.1	12.71	3.4	2.90	6.4
430	Durbanville	2005	11	0.41	0.79	7.2	3.7	13.67	1.8	3.47	6.8
431	Cape Agulhas	2005	7.4	0.20	0.38	5.1	2.7	14.56	4.3	3.33	6.5
432	Stellenbosch	2005	3.2	0.18	NQ	-	5.6	13.49	4.3	3.45	6.1
433	Stellenbosch	2005	5.8	0.29	0.22	3.8	5.0	12.71	1.6	3.06	7.2
434	Stellenbosch	2005	2.7	0.19	NQ	-	7.0	13.32	3.6	3.23	6.6
435	Wellington	2005	1.1	0.11	ND	-	10.0	13.06	1.5	3.42	4.8
436	Western Cape	2005	3.5	0.13	0.13	3.7	3.7	12.19	2.0	3.24	6.4
437	Wellington	2005	0.72	0.12	NQ	-	16.7	11.93	1.5	3.11	6.3
438	Western Cape	2005	0.62	0.16	ND	-	25.8	12.10	4.4	3.50	6.2
440	Western Cape	2005	2.1	0.14	NQ	-	6.7	12.97	1.6	3.67	5.5
441	Stellenbosch	2005	4.4	0.19	0.14	3.2	4.3	12.88	1.3	3.25	7.5
442	Elim	2005	11	0.24	0.73	6.6	2.2	14.56	3.6	3.17	7.1
443	Western Cape	2005	0.52	0.14	NQ	-	26.9	14.02	3.1	3.60	5.9
444	Stellenbosch	2005	6.3	0.17	0.28	4.4	2.7	13.41	2.8	3.42	6.8
445	Western Cape	2005	2.2	0.20	0.18	8.2	9.1	12.19	2.2	3.38	6.3
446	Darling	2005	14	0.49	0.96	6.9	3.5	12.45	1.7	3.72	5.3
447	Western Cape	2005	0.54	0.12	NQ	-	22.2	12.80	2.6	3.58	4.3
448	Stellenbosch	2005	8.2	0.20	0.40	4.9	2.4	14.38	2.0	3.14	7.8

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
449	Constantia	2005	12	0.73	0.76	6.3	6.1	13.14	1.5	3.17	6.2
450	Coastal region	2005	5.7	0.39	0.32	5.6	6.8	12.88	1.4	3.29	7.1
451	Stellenbosch	2005	1.8	NQ	NQ	-	-	13.85	1.0	3.27	6.0
452	Elim	2005	14	0.30	0.72	5.1	2.1	13.14	2.0	3.37	6.6
453	Stellenbosch	2005	9.1	0.33	0.40	4.4	3.6	12.36	5.2	3.11	8.3
454	Franschhoek	2005	0.96	NQ	NQ	-	-	13.76	1.6	3.33	6.2
455	Western Cape	2005	3.7	0.13	NQ	-	3.5	11.51	2.3	3.32	6.9
456	Coastal region	2005	4.2	0.29	0.20	4.8	6.9	12.88	1.2	3.34	7.0
457	Coastal region	2005	16	0.39	0.63	3.9	2.4	13.94	3.6	3.36	7.4
458	Coastal region	2005	5.7	0.33	0.41	7.2	5.8	12.88	4.0	3.20	6.4
459	Elgin	2004	3.6	0.13	0.21	5.8	3.6	12.97	1.7	3.16	7.5
460	Stellenbosch	2005	2.3	NQ	NQ	-	-	12.97	3.3	3.19	7.1
461	Constantia	2005	9	0.56	0.43	4.8	6.2	13.41	2.4	3.30	6.4
462	Western Cape	2004	2.7	0.18	ND	-	6.7	12.10	1.3	3.17	6.4
463	Coastal region	2005	4.8	0.17	0.13	2.7	3.5	12.80	1.8	3.31	6.3
464	Western Cape	2005	0.54	NQ	NQ	-	-	12.28	1.6	3.60	5.3
465	Franschhoek	2005	7.8	0.28	0.50	6.4	3.6	13.85	2.5	3.42	6.9
466	Paarl	2005	7.3	0.20	0.28	3.8	2.7	13.06	1.4	3.65	6.4
467	Walker Bay	2005	8.4	0.25	0.30	3.6	3.0	13.06	1.7	3.04	6.8
468	Durbanville	2003	0.74	0.18	NQ	-	24.3	12.02	1.7	3.56	5.2
469	Western Cape	2005	2.9	0.15	0.12	4.1	5.2	12.36	3.1	3.27	5.9
470	Stellenbosch	2005	3.9	0.11	0.20	5.1	2.8	14.65	6.7	3.14	6.6
471	Coastal region	2005	7.7	0.29	0.26	3.4	3.8	12.36	4.4	3.10	7.3
472	Western Cape	2005	1.3	0.13	NQ	-	10.0	12.62	1.5	3.56	6.4
473	Coastal region	2005	4.6	0.17	0.13	2.8	3.7	12.88	1.5	3.28	6.3
474	Western Cape	2004	1.7	0.13	NQ	-	7.6	12.10	1.2	3.26	6.3
475	Stellenbosch	2005	6.1	0.13	0.22	3.6	2.1	13.14	2.1	2.99	6.5
476	Western Cape	2005	0.48	NQ	NQ	-	-	12.97	1.7	3.31	7.4
477	Coastal region	2005	4.4	0.24	0.14	3.2	5.5	13.58	2.6	3.10	7.4
478	Stellenbosch	2005	2	0.14	0.14	7.0	7.0	12.71	2.9	3.46	6.3
479	Constantia	2005	7.3	0.29	0.43	5.9	4.0	12.53	3.8	3.11	7.6
480	Paarl	2004	2.4	0.17	0.16	6.7	7.1	14.38	3.0	3.68	5.2
481	Stellenbosch	2004	11	0.45	1.1	10.0	4.1	12.53	5.0	3.36	6.7
482	Groenekloof	2005	15	0.55	0.98	6.5	3.7	12.80	1.3	3.40	7.1
483	Durbanville	2003	2.9	0.17	0.17	5.9	5.9	12.19	2.3	3.31	5.9
485	Western Cape	2005	7.6	0.25	0.36	4.7	3.3	13.14	2.6	3.16	7.1

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
487	Stellenbosch	2005	6.7	0.28	0.37	5.5	4.2	14.47	1.5	3.40	6.0
489	Simonsberg	2005	5	0.20	0.17	3.4	4.0	12.97	2.0	3.33	7.0
490	Durbanville	2004	8.7	0.32	0.44	5.1	3.7	13.32	1.8	3.40	6.6
491	Western Cape	2004	2.6	NQ	0.13	5.0	-	13.94	3.6	3.41	6.7
492	Coastal region	2006	17	0.39	0.72	4.2	2.3	13.23	2.8	3.32	6.9
493	Robertson	2006	2.1	ND	NQ	-	-	12.19	2.3	3.28	7.0
494	Stellenbosch	2005	3.3	0.32	0.15	4.5	9.7	14.56	4.0	3.31	6.3
495	Coastal region	2005	1.8	NQ	0.34	18.9	1.7	11.59	2.9	3.27	5.9
496	Coastal region	2004	4.6	NQ	0.22	4.8	-	13.58	1.4	3.25	6.6
497	Coastal region	2004	11	0.26	0.66	6.0	2.4	12.28	1.9	3.44	6.2
498	Western Cape	2004	3.7	NQ	NQ	-	-	11.93	6.2	3.44	6.8
499	Western Cape	2004	7.2	0.13	0.45	6.3	1.8	13.23	3.4	3.31	6.5
500	Western Cape	2005	40	0.78	2.4	5.9	2.0	15.19	2.0	3.54	5.8
501	Elgin	2006	6	0.18	0.41	6.8	3.0	12.80	2.3	3.36	6.7
502	Paarl	2005	0.54	0.26	NQ	-	48.1	12.28	1.5	3.69	5.0
503	Western Cape	2005	1.1	NQ	NQ	-	-	13.49	3.5	3.54	6.1
504	Wellington	2005	0.6	ND	NQ	-	-	12.02	1.2	3.25	6.5
505	Stellenbosch	2005	5.4	NQ	0.16	3.0	-	13.76	2.2	3.12	7.4
506	Constantia	2005	12	2.0	1.2	10.2	16.3	13.23	2.1	3.25	7.1
507	Stellenbosch	2005	8.4	0.14	0.48	5.7	1.7	12.10	5.0	3.07	8.0
508	Stellenbosch	2005	7.4	0.22	0.47	6.4	3.0	13.67	1.8	3.42	6.2
509	Western Cape	2004	2.7	NQ	0.14	5.2	-	11.76	1.6	3.25	7.1
510	Stellenbosch	2005	2.6	NQ	NQ	-	-	13.67	2.6	3.17	5.9
511	Constantia	2005	7	0.26	0.36	5.1	3.7	12.97	2.6	3.15	7.5
512	Western Cape	2005	4.3	0.12	0.24	5.6	2.8	12.80	2.9	3.29	6.9
513	Western Cape	2005	3.4	NQ	0.11	3.2	-	12.53	1.6	3.48	6.3
514	Stellenbosch	2004	1.3	ND	NQ	-	-	13.49	6.2	3.62	5.5
515	Western Cape	2004	9.8	0.25	1.1	10.7	2.6	12.02	1.9	3.91	5.3
516	Stellenbosch	2004	8.4	0.23	0.45	5.4	2.7	12.19	4.0	3.43	6.2
517	Elgin	2005	13	0.26	0.88	6.8	2.0	13.06	1.2	3.37	5.6
518	Western Cape	2005	43	1.0	3.3	7.7	2.3	15.19	2.0	3.54	5.8
519	Coastal region	2005	5	0.18	0.20	4.0	3.6	14.11	4.1	3.29	6.6
520	Western Cape	2004	6.7	0.16	0.49	7.3	2.4	12.62	4.1	3.22	5.4
521	Simonsberg	2005	5.2	NQ	0.13	2.5	-	13.14	2.2	3.27	6.8
522	Simonsberg	2005	4.3	0.36	0.14	3.3	8.4	12.02	1.4	3.26	6.8
523	Stellenbosch	2005	3.8	NQ	0.15	3.9	-	13.32	1.9	3.22	6.5

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
524	Constantia	2002	6.1	0.13	0.43	7.0	2.1	14.11	80.6	3.90	7.8
525	Coastal region	2005	5.7	0.13	0.28	4.9	2.3	14.11	2.9	3.28	6.1
526	Elgin	2006	17	0.66	0.76	4.5	3.9	13.32	1.1	3.03	7.1
527	Stellenbosch	2004	1.2	NQ	NQ	-	-	13.41	4.1	3.16	6.8
528	Cederberg	2004	3.3	NQ	NQ	-	-	13.06	1.6	3.24	7.3
529	Western Cape	2005	1.2	NQ	ND	-	-	12.80	2.7	3.29	6.9
531	Stellenbosch	2005	13	0.24	0.38	2.9	1.8	12.36	1.7	3.34	6.0
532	Coastal region	2005	2.2	NQ	NQ	-	-	12.71	2.1	3.37	5.9
533	Western Cape	2005	5.8	0.16	0.29	5.0	2.8	13.41	3.5	3.34	6.1
534	Western Cape	2005	11	0.18	0.59	5.4	1.6	13.41	1.8	3.24	6.3
535	Stellenbosch	2006	10	0.41	0.32	3.2	4.1	11.76	5.4	3.31	7.0
536	Western Cape	2006	1.3	NQ	NQ	-	-	12.62	2.1	3.48	5.5
537	Western Cape	2005	4.5	NQ	0.20	4.4	-	12.53	3.4	3.24	5.5
538	Western Cape	2005	6.1	0.14	0.46	7.5	2.3	12.97	4.0	3.16	7.5
539	Stellenbosch	2004	12	0.41	0.87	7.3	3.4	12.45	1.5	3.24	6.7
540	Stellenbosch	2005	9.8	0.66	0.41	4.2	6.7	13.67	2.2	3.30	6.7
541	Western Cape	2006	1.3	NQ	ND	-	-	12.28	1.7	3.25	6.2
542	Western Cape	2006	4.6	0.18	0.34	7.4	3.9	13.06	3.9	3.43	5.6
543	Stellenbosch	2005	16	0.41	0.94	5.9	2.6	13.14	2.5	3.24	6.4
544	Cape Point	2005	11	0.24	0.45	4.1	2.2	13.23	2.5	3.17	6.6
545	Stellenbosch	2005	6.5	0.16	0.40	6.2	2.5	13.41	3.4	3.38	7.6
546	Western Cape	2005	1.9	NQ	ND	-	-	12.88	4.8	3.25	8.4
547	Stellenbosch	2005	5.5	0.14	0.31	5.6	2.5	12.80	1.9	3.35	6.9
549	Western Cape	2005	5.7	0.11	0.41	7.2	1.9	12.53	3.0	3.31	7.6
550	Franschhoek	2005	1.6	ND	ND	-	-	13.32	4.0	3.14	5.6
551	Western Cape	2005	7.6	NQ	0.14	1.8	-	12.28	1.3	3.30	6.2
552	Stellenbosch	2004	4.1	NQ	0.17	4.1	-	13.06	1.8	3.26	6.2
553	Stellenbosch	2004	4.6	0.11	0.25	5.4	2.4	12.53	5.2	3.21	7.0
554	Stellenbosch	2005	5.6	0.27	0.30	5.4	4.8	14.20	1.8	3.25	6.1
555	Western Cape	2005	5.4	0.14	0.36	6.7	2.6	12.88	3.0	3.37	6.0
556	Coastal region	2005	3.7	0.12	0.13	3.5	3.2	12.36	2.4	3.18	7.2
557	Western Cape	2006	12	0.29	0.43	3.6	2.4	13.85	2.1	3.09	6.3
558	Robertson	2007	2	0.13	ND	-	6.5	11.76	1.0	3.31	6.3
559	Bottelary	2005	4.2	NQ	NQ	-	-	13.41	1.8	3.10	6.0
560	Durbanville	2005	11	0.16	0.45	4.1	1.5	13.32	2.8	3.30	6.1
561	Western Cape	2005	38	0.81	2.4	6.3	2.1	15.19	1.7	3.42	6.8

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
562	Constantia	2005	13	1.1	1.1	8.7	8.2	13.67	2.6	3.45	6.5
563	Western Cape	2005	10	0.21	0.48	4.8	2.1	13.23	1.1	3.30	6.3
564	Stellenbosch	2005	1.7	NQ	ND	-	-	13.23	2.8	3.34	6.7
565	Stellenbosch	2005	5.5	0.14	0.32	5.8	2.5	13.14	3.3	3.39	7.6
566	Stellenbosch	2005	6	0.11	0.39	6.5	1.8	12.36	6.1	3.35	6.4
567	Western Cape	2004	2.6	NQ	0.17	6.5	-	13.67	5.0	3.44	5.8
568	Coastal region	2005	14	0.38	0.81	5.8	2.7	13.67	2.0	3.22	6.7
569	Western Cape	2007	18	0.42	1.1	6.2	2.3	12.62	1.4	2.96	7.7
570	Western Cape	2005	5.4	NQ	0.21	3.9	-	12.36	4.0	3.28	6.7
571	Durbanville	2005	12	0.20	0.44	3.7	1.7	11.68	1.2	3.40	6.0
572	Franschhoek	2005	1.1	NQ	NQ	-	-	14.65	1.3	3.32	5.9
573	Western Cape	2005	3.6	NQ	0.24	6.7	-	12.62	1.7	3.36	6.2
574	Stellenbosch	2005	8.4	0.21	0.49	5.8	2.5	13.94	2.2	3.26	7.7
576	Simonsberg	2005	5.9	0.12	0.32	5.4	2.0	12.71	1.5	3.30	7.1
577	Stellenbosch	2004	4.2	NQ	0.20	4.8	-	12.53	1.5	3.36	6.4
578	Cape Agulhas	2005	4.9	NQ	0.38	7.8	-	12.62	2.2	3.36	5.8
579	Simonsberg	2005	8.1	0.21	0.55	6.8	2.6	13.76	1.6	3.35	6.3
580	Stellenbosch	2005	3.9	0.11	0.32	8.2	2.8	13.58	1.5	3.40	5.7
581	Cape Agulhas	2005	13	0.23	0.82	6.3	1.8	13.06	2.0	3.26	6.5
582	Coastal region	2005	7.7	NQ	0.20	2.6	-	12.71	3.4	3.42	6.5
583	Elim	2005	9.5	0.28	0.73	7.7	2.9	13.58	2.9	3.28	6.7
584	Walker Bay	2005	1.2	NQ	0.13	10.8	-	13.58	1.6	3.33	6.4
585	Coastal region	2005	3.7	NQ	0.14	3.8	-	12.02	2.3	3.22	6.3
586	Paarl	2005	20	NQ	0.18	0.9	-	13.41	2.3	3.44	6.6
587	Western Cape	2005	5.3	0.11	0.16	3.0	2.1	12.19	3.6	3.31	5.8
588	Stellenbosch	2004	20	0.26	0.65	3.3	1.3	12.45	3.4	3.51	6.5
589	Western Cape	2005	44	0.83	3.9	9.0	1.9	15.19	2.0	3.60	5.9
590	Western Cape	2006	24	0.67	2.1	8.7	2.8	12.80	3.4	3.27	6.6
593	Coastal region	2005	4.1	NQ	NQ	-	-	12.10	2.6	3.22	6.0
594	Simonsberg	2005	4.9	0.11	0.16	3.3	2.2	13.58	2.5	3.40	6.4
595	Constantia	2005	9.1	0.51	0.40	4.4	5.6	13.06	2.6	3.30	2.6
596	Paarl	2005	0.78	NQ	NQ	-	-	13.06	2.8	3.44	5.4
597	Coastal region	2005	2.9	NQ	NQ	-	-	12.88	4.7	2.97	6.8
598	Bottelary	2005	7	0.26	0.45	6.4	3.7	13.76	2.7	3.21	6.8
599	Western Cape	2006	23	0.51	1.5	6.3	2.2	12.8	3.7	3.28	6.7
600	Robertson	2007	5.7	0.12	0.32	5.6	2.1	12.42	0.7	3.60	8.1

No.	Origin	Vintage	IBMP(ng/L)	SBMP(ng/L)	IPMP(ng/L)	IPMP(%)	SBMP(%)	Alc(%v/v)	Sug(g/L)	pH	T.A.(g/L)
601	Durbanville	1999	16	0.43	1.8	11.4	2.7	12.93	2.0	3.53	7.4
602	Durbanville	2000	6.4	NQ	0.26	4.1	-	12.98	2.5	3.45	6.5
603	Durbanville	2001	38	0.69	2.5	6.6	1.8	13.00	2.8	3.22	7.5
604	Durbanville	2002	20	0.60	2.2	10.8	3.0	12.76	1.8	3.32	7.7
605	Durbanville	2003	14	0.35	1.5	11.0	2.5	12.57	1.8	3.42	7.2

a = adulterated sample 1

b = adulterated sample 2

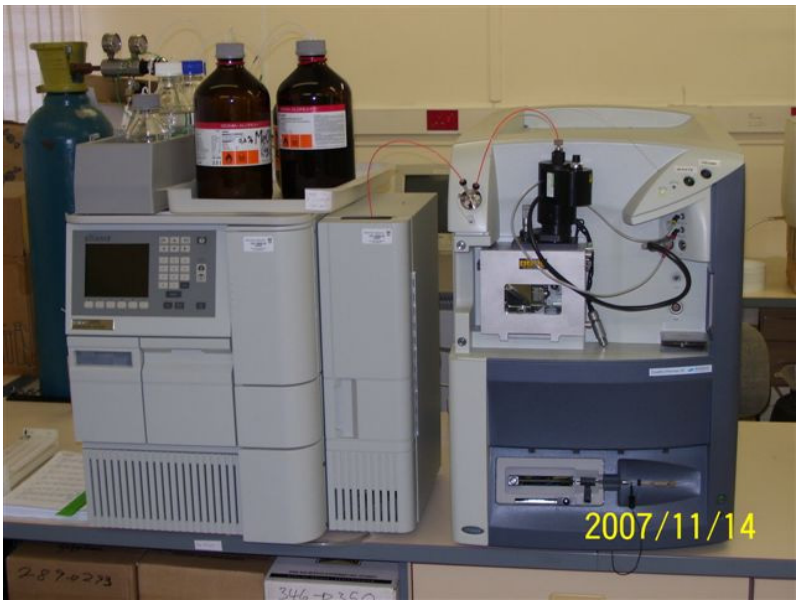
ND = not detected

NQ = not quantified

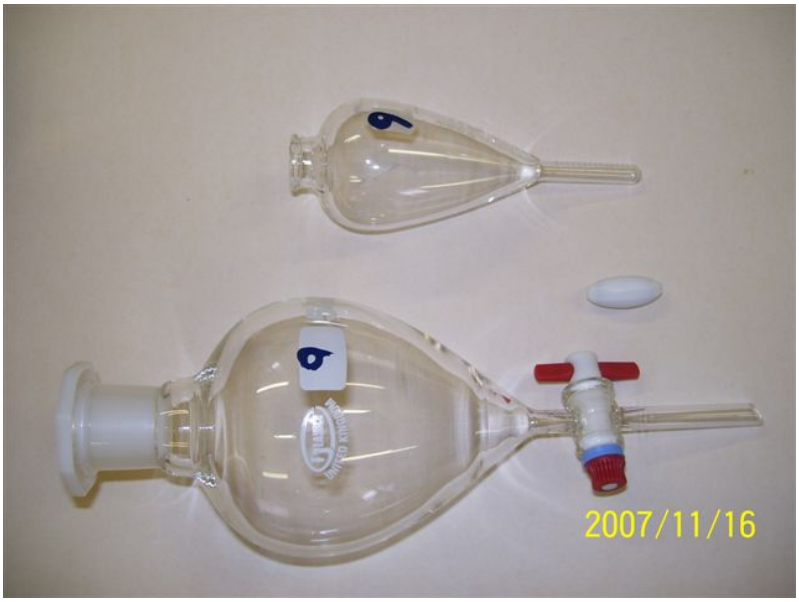
Table B: Data pertaining to wine of other cultivars (secondary information).

No	Cultivar	Origin	Vintage	IBMP (ng/L)
A1	Pinotage	Western Cape	2006	0.90
A2	Pinotage	Stellenbosch	2004	0.78
A3	Pinotage	Klein Rivier	2006	3.1
A4	Pinotage	Western Cape	2003	1.1
A5	Pinotage	Western Cape	2004	1.7
A6	Pinotage	Western Cape	2006	1.3
B1	Chardonnay	Western Cape	2006	0.66
B2	Chardonnay	Coastal region	2006	0.46
B3	Chardonnay	Western Cape	2007	0.30
B4	Chardonnay	Western Cape	2006	1.0
B5	Chardonnay	Robertson	2007	0.20
B6	Chardonnay	Western Cape	2007	0.56
C1	Cabernet Sauvignon	Paarl	2003	7.2
C2	Cabernet Sauvignon	Elgin	2005	3.4
C3	Cabernet Sauvignon	Stellenbosch	2003	12
C4	Cabernet Sauvignon	Stellenbosch	2006	8.8
C5	Cabernet Sauvignon	Western Cape	2006	13
C6	Cabernet Sauvignon	Stellenbosch	2004	17
D1	Shiraz	Klein Rivier	2005	1.5
D2	Shiraz	Coastal region	2004	3.4
D3	Shiraz	Tradauw	2005	2.7
D4	Shiraz	Elim	2007	2.4
D5	Shiraz	Western Cape	2005	2.2
D6	Shiraz	Robertson	2006	2.4
E1	Chenin Blanc	Western Cape	2006	0.38
E2	Chenin Blanc	Western Cape	2007	1.1
E3	Chenin Blanc	Paarl	2007	0.48
E4	Chenin Blanc	Coastal region	2006	0.38
F1	Merlot	Experimental wine		46
F2	Merlot	Experimental wine		52
F3	Merlot	Experimental wine		34
F4	Merlot	Experimental wine		46
F5	Merlot	Experimental wine		32
F6	Merlot	Experimental wine		56
F7	Merlot	Experimental wine		26
F8	Merlot	Experimental wine		34
F9	Merlot	Experimental wine		36
F10	Merlot	Experimental wine		26
F11	Merlot	Experimental wine		50
F12	Merlot	Experimental wine		48
F13	Merlot	Experimental wine		46
F14	Merlot	Experimental wine		52
F15	Merlot	Western Cape	2005	6.9
F16	Merlot	Western Cape	2004	7.7

Appendix 3: Pictures



The LC-MS system utilized in the study.



Separating funnel, 35 mm egg-shaped magnet and calibrated pear-shaped flask.

Distillation apparatus with 60 cm fractionating column.

Equipment for evaporation of solvent from sample extracts.