Application of comprehensive two-dimensional gas chromatography to wine analysis

by

Jochen Vestner

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Supervisor: Dr. A.J. de Villiers
Co-supervisor: Dr. A.G.J. Tredoux
Faculty of Science
Department of Chemistry and Polymer Science

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Summary

This study focused on the potential of comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS) for the improved analysis of volatile wine constituents. Solid phase microextraction (SPME) in combination with GC×GC-TOF-MS was successfully used for the detailed investigation of the impact of three commercial *Oenococcus oeni* lactic acid bacteria (LAB) strains on the volatile composition of Pinotage wines subjected to malolactic fermentation (MLF). Due to increased separation power and enhanced sensitivity obtained by using two orthogonal separations coupled with the structural information provided by deconvoluted TOF-MS spectra, GC×GC-TOF-MS allowed for the identification and semi-quantitative analysis of much larger numbers of compounds compared to previous studies applying one-dimensional gas chromatography. The combination of univariate and multivariate statistical assessment was used as a powerful tool for data interpretation. The obtained results contribute significantly to the understanding of the impact of MLF on the volatile composition of Pinotage wine Some compounds have been linked to MLF for the first time.

Moreover, the impact of these commercial starter cultures on the composition of volatile sulfur and nitrogen compounds in the same wines was studied by one-dimensional gas chromatographic methods with headspace injection and solid supported liquid-liquid extraction together with sulfur selective detection and tandem mass spectrometry. This study demonstrated also for the time, the impact of MLF on the composition of volatile sulfur and nitrogen compounds in Pinotage wine.

GC×GC-TOF-MS was further used for the evaluation of the suitability of a new phase for stir bar sorptive extraction (SBSE) analysis of wine volatiles. Despite instrumental complications, beneficial extraction properties of the new stir bar phase for especially more polar compounds could be demonstrated. In addition, the extraction ability of this novel phase was evaluated for the analysis of selected thiazoles in wine using heart-cutting two dimensional gas chromatography in combination with nitrogen selective detection. Advantageous extraction performance of the new stir bar phase compared to a conventional polydimethylsiloxane (PDMS) phase for the determined thiazoles was demonstrated.

Opsomming

Hierdie studie het gefokus daarop om die potensiaal van omvattende twee-dimensionele gaschromatografie gekombineer met vlugtyd massaspektrometrie (GC×GC-TOF-MS) vir die verbeterde analise van vlugtige wynkomponente te ondersoek. Soliede fase mikro-ekstraksie (SPME) in kombinasie met GC×GC TOF MS is met sukses aangewend vir ‘n ondersoek na die impak van drie kommersiële *Oenococcus oeni* melksuur bakteria (LAB) rasse op die samestelling van die vlugtige fraksie van Pinotage wyne wat appelmelksuurgisting (AMG) ondergaan het. As gevolg van die verbeterde skeidingsvermoë en die verhoogte sensitiwiteit wat verkry word deur twee ortogonale skeidings te kombineer, tesame met die inligting aangaande die molekulêre struktuur wat die die gedekonvoleerde TOF massaspektra verskaf, maak GC×GC-TOF-MS die identifikasie en semi-kwantitatiewe analise van aansienlik meer komponente, in vergelyking met die gebruik van een-dimensionele gaschromatografie, moontlik.

Die kombinasie van monoveranderlike asook multiveranderlike statistiese evaluering is gebruik as ‘n kragtige tegniek vir data interpretasie. Die resultate wat verkry is dra tot ‘n groot mate by tot die ontrafeling en begrip aangaande die impak wat AMG op die samestelling van vlugtige komponente in Pinotage wyn het. Daar word ook vir die eerste keer aangetoon dat somminge komponente verband te hou met AMG.

Aanvullend hiertoe is die impak wat hierdie kommersiële kulture (wat gebruik word om fermentasie te inisieer) op die voorkoms van swawel en stikstof bevattende vlugtige komponente het bestudeer deur gebruik te maak van een-dimensionele gaschromatografiese metodes met ‘headspace’ inspuiting en vloeistof-voeistof ekstraksie tesame met swawel en stikstof selektiewe deteksie en tandem massaspektrometrie. Hierdie ondersoek werp lig, ook vir die eerste keer, op die samestelling van vlugtige swawel en stikstof bevattende komponente in Pinotage wyn.

GC×GC-TOF-MS is ook gebruik vir die evalueering van die toepaslikheid van ‘n nuwe stasionêre fase vir gebruik met roerstaaf sorptiewe ekstraksie (SBSE) vir die analisering van vlugtige komponente in wyn. Ten spyte van instrumentele komplikasies, is die voordele wat hierdie nuwe fase vir die ekstraksie van vernaamlik meer polêre komponete aangetoon. Vervolgens is die ekstraksievermoë van hierdie nuwe fase vir die analise van sekere tiasole in wyn met ‘heart-cutting’ twe-dimensionaly gaschromatografie in kombinasie met stikstof-selektiewe deteksie

gedemonstreer. Verbeterde ekstraksie van die nuwe roerstaaf fase vir die analise van tiasole, in vergelyking met ‘n tradisionele polydimethylsiloxane (PDMS) fase is voorts aangetoon.

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Abbreviations

2AAP	2-Aminoacetophenone
ADS	Alkyl-diol-silica
AED	Atomic emission detector
ANOVA	Analysis of variance
ASE	Accelerated solvent extraction
BHT	Butylated hydroxyl toluene
BTH	Benzothiazole
CAD	Collision activated decomposition
CAR	Carboxen™
CE	Capillary electrophoresis
CID	Collision induced dissociation
CI	Chemical ionisation
CW	Carbowax
DC	Direct current
DCM	Dichloromethane
DMS	Dimethyl sulfide
DVB	Divinyl benzene
ECD	Electron capture detector
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
EPC	Electronic pneumatic control
FFAP	Free fatty acid phase
FID	Flame ionisation detector
FPD	Flame photometric detector
GC	Gas chromatography
GC×GC	Comprehensive two-dimensional gas chromatography
GC-GC	Heart-cutting two-dimensional gas chromatography
HPLC	High performance liquid chromatography
HS-SPME	Headspace SPME
HSSE	Headspace sorptive extraction
IAA	Indole-3-acetic acid
i.d.	Inner diameter
IS	Internal standard
K_D	Distribution coefficient
$K_{O/W}$	Octanol-water partitioning coefficient
$K_{PDMS/W}$	PDMS-water partitioning coefficient

LD	Liquid desorption
LLE	Liquid-liquid extraction
LRI	Linear retention index
LSD	Fisher's Least Significant Difference
LVI	Large volume injection
µLLE	Micro liquid-liquid extraction
MASE	Microwave assisted extraction
mg/L	Milligram per liter
MIP	Molecular imprinted polymers
MLF	Malolactic fermentation
MPS	MultiPurpose Sampler
MS	Mass spectrometry
NCD	Nitrogen chemiluminescence detector
ng/L	Nanogram per liter
NIST	National Institute of Standards
NPD	Nitrogen phosphorus detector
OTT	Open tubular trap
PA	Polyacrylate
PC	Principle component
PCA	Principle component analysis
PDMS	Polidimethylsiloxane
PFPD	Pulsed flame photometric detector
PLOT	Porous layer open tubular
PPESK	Poly(phthalazine) ether sulfone ketone
PPY	Polypyrrole
PU	Polyurethane
pg/L	Picogram per liter
ppm	Parts per million
PTV	Programmed temperature vapourisation
QIT	Quadrupole ion trap detection (mass spectrometry)
qMS	Quadrupole mass spetrometry
RAM	Restricted access materials
RF	Radio frequency
RI	Retention index
RSD	Relative standard deviation
SBSE	Stir bar sorptive extraction
SDVB	Styrene divinyl benzene
SMM	S-methyl methionine

SIM	Selected ion monitoring
SLE	Solid supported liquid-liquid extraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
SCD	Sulphur chemiluminescence detector
TD	Thermal desorption
TDS	Thermal desorption system
TDU	Thermal desorption unit
TIC	Total ion current
TOF	Time-of-flight
UAE	Ultrasonic assisted extraction
VSC	Volatile sulfur compounds
VP	Vinylpyridine
VPL	Vinylpyrrolidone
VI	Vinylimidazole
WCOT	Wall Coated Open Tubular
WAX	Polyethylene glycol
µLLE	Micro liquid-liquid extraction

Symbols:

α	Selectivity factor
β	Phase ratio
d_f	Film thickness
k	Retention factor
$K_{O/W}$	Octanol/water partition coefficient
N	Plate number
n	Peak capacity
m_{SBSE}	Mass of analyte in the SBSE phase
m_W	Mass of analyte in the water phase
m_0	Total mass of analyte in the sample prior to extraction
R_S	Chromatographic resolution
T_G	Glass transition point
t_n	Retention time of the last eluted compound
t_0	Void time
I^T	Programmed-temperature retention index

$I^{St.phase}$	Isothermal retention index for a stationary phase

1

General introduction and objectives

1.1 General introduction

Over 6000 years ago *Vitis vinifera* (the common grape vine) was already cultivated and wine produced in Mesopotamia in the fertile land between the Tigris and Euphrates rivers known as the cradle of civilization. Winemaking has significantly changed since this time and vinification practices have evolved mainly based on changes in consumer demands. The most important consumer requirement pertains to the sensory properties of wine, which in turn are strongly determined by its aroma. To improve wine quality and meet consumer expectations a greater understanding of the formation and alteration of wine aroma is necessary. From the grape in the vineyard to the wine in the consumer's glass many different processes affect the flavor and style of a wine. Factors affecting the wine flavor in the vineyard are for instance the climate, canopy management, water management, harvesting time and others. Not less important are post-harvesting processes such as alcoholic fermentation, malolactic fermentation (MLF) and barrel aging; the latter two are more often performed during red wine production. To achieve an in-depth knowledge of these processes and the factors affecting them, a rigorous scientific approach is necessary. In this field of research advanced analytical instrumentation plays an essential role.

For example, our current knowledge about the ancient vinification process is based on the study of the volatile and semi-volatile compounds in oenological residues of ancient pottery from Egypt[1]. Application of cutting-edge analytical methods such as gas chromatography and high performance liquid chromatography hyphenated with mass spectrometry showed that wines at the court of the Pharaoh's were often enriched with resin and herbs. Nowadays wine is no longer flavored in this manner (in fact, wine is considered a natural product and addition of extraneous substances is forbidden).

From a chemical point of view the aroma impression of wine is a result of the detection of volatile constituents by the human nose. The volatile composition of wine is therefore a crucial quality marker. However, the analysis of wine volatiles is far from straightforward due to the complexity of the wine matrix, which contains high levels of ethanol, organic acids, sugars, tannins and over 700 different volatile compounds. Highly sophisticated analytical methods are therefore required to manage such a difficult task.

Gas chromatography is the most common analytical method for the analysis of volatile compounds; the aforementioned application clearly demonstrates the potential of this technique. Though powerful, conventional one-dimensional capillary gas chromatography

[1] McGovern, P. E.; Mirzoian, A.; Hall, G. R. Ancient Egyptian herbal wines. *Proceedings of the National Academy of Sciences of the United States of America* **2009**, vol. 106, no. 18, 7361-8366

does show limitations when it comes to the analysis of highly complex mixtures such as wine.

One way of overcoming the limitations of conventional GC for complex samples is to use multidimensional chromatography, where improved resolution is realized by subjecting a sample to two independent separation processes such as comprehensive two-dimensional gas chromatography (GC×GC). GC×GC is one of the younger multidimensional techniques which has been shown to be a particularly powerful method for the analysis of complex mixtures of volatiles, especially when used in combination with time-of-flight mass spectrometry (TOF-MS). However, this technology has to date been used sparingly for the analysis of wine volatiles. Reasons for this include a more complex instrument set-up which is costly and requires highly skilled operators, especially concerning data processing after the analysis.

Prior to a gas chromatographic separation, sample preparation plays a crucial role in removing interfering matrix constituents and improving sensitivity by selective enrichment of the analytes of interest. This step therefore simplifies the chromatographic analysis of specific compounds. However, there is no universal form of sample pre-treatment generically suitable for the untargeted screening of the diverse wine volatiles, since the concentration range of aroma-active wine compounds spans mg/L to sub-ng/L levels and cover a wide polarity range. These facts have led to the development of numerous sample pretreatment techniques for wine volatile analysis, which are sensitive but also environmentally friendly; especially for polar compounds in wine.

An alternative approach to simplify analysis of complex samples is the use of element specific detectors. The large number of volatile compounds in wine frequently leads to co-elution in one-dimensional gas chromatography. Selective detectors provide the possibility to record element specific traces (e.g. for sulfur and nitrogen containing compounds), thereby reducing the demands placed on chromatographic separation.

1.2 Objectives of this study

The principle objective of this study was to investigate the potential of GC×GC-TOF-MS to improve the analysis of wine volatiles. In order to address a relevant topic of interest in wine research GC×GC-TOF-MS was applied to study the effect of malolactic fermentation on the volatile composition of the uniquely South African grape variety, Pinotage. Experimental wines fermented using different commercial starter cultures under controlled conditions were used in this study. These wine samples allowed the evaluation of the separation power of GC×GC and the potential to identify compounds which have not previously been linked to

MLF. As GC×GC provided no information about changing levels of volatile sulfur compounds, a supplementary study using one-dimensional gas chromatography with selective detectors was conducted on the same wines. The second major aim of this thesis was the evaluation of alternative sample preparation techniques in combination with GC×GC for the analysis of wine volatiles. For this purpose a newly available more polar phase for stir bar sorptive extraction (SBSE) was used. The application of this SBSE phase for extraction of volatiles before two-dimensional heart-cutting gas chromatography (GC-GC) with nitrogen chemiluminescence detection for the analysis wine thiazoles was also explored.

2

Gas chromatographic separation

2.1 One dimensional gas chromatography

The term chromatography refers to a broad range of separation methods based on the distribution of substances between two non-miscible phases, where one phase is in motion (mobile phase) and the other is static (stationary phase). In the case of capillary gas chromatography (GC), volatile and semi-volatile compounds are separated by differential partitioning between a gaseous mobile phase and a (mostly) liquid stationary phase. As mobile phase different carrier gases such as helium, hydrogen and in some cases nitrogen are commonly used. The carrier gas transports a gaseous sample through a column coated on the inside with a thin film of the stationary phase. Partitioning of compounds between the stationary phase and the mobile phase depends on temperature and on the physiochemical properties of analytes and the stationary phase. Analytes with higher affinity for the stationary phase are retained longer in the column, whereas compounds with lower affinity elute earlier (*1*, *2*).

In addition to stationary phase interactions, in GC, analytes are separated according to their vapor pressures; therefore the separation is a function of temperature. Separations can be carried out isothermally or with a programmed temperature gradient. However, isothermal separation is hardly used as temperature programming poses significant advantages, such as added versatility in complex sample analysis and narrower peaks for later eluting compounds (with higher boiling points) resulting in better sensitivity.

A GC instrument consists principally of an injector for introduction of samples in the system, the column, which is placed in a temperature controlled oven and is responsible for separation of the analytes in the sample, and the detector which detects the separated compounds as they elute from the column. These parts will be briefly discussed below.

2.1.1 GC columns

The column is often described as the ‘heart’ of any chromatographic system. In GC two different types of columns are used: columns packed with solid supported particles coated with the stationary phase or adsorbent (packed columns), and open tubular columns with a stationary phase film on the inner wall (capillary columns). Packed columns are made of metal or glass with outside diameters of 1/4” (3.2 cm) to 1/8” (6.4 cm), whereas capillary columns are made of fused silica with inner diameters of 0.1 to 0.5 mm. Since the work of Golay (*3*), capillary columns have largely replaced packed columns, except for specialized applications such as gas analysis. Capillary columns provide a significant increase in resolution compared to packed columns due to their small internal diameter and coating on the inner wall, which leads to better mass transfer across shortened diffusion distances.

Moreover, long capillary columns can be operated at realistic gas pressures in contrast to packed columns with larger diameters (*4*). Therefore, capillary columns are well established today, whereas packed columns are only, as mentioned earlier, used for special applications.

The capillary columns most commonly used are between 30 and 60 m long, with internal diameters of 0.25 to 0.32 mm and film thicknesses of 0.1 µm to 5 µm. The selection of a column is, however, highly dependent on the physiochemical properties of the target analytes and the complexity of the sample. The choice of capillary column for a certain application is often made according to the following priority: stationary phase, internal diameter, film thickness and lastly length. The two basic types of capillary columns are wall coated open tubular (WCOT) columns and porous layer open tubular (PLOT) columns, of which WCOT columns are used most frequently. The following discussion focuses only on WCOT columns; the type of column used in the current study.

A wide range of different stationary phases are commercially available, varying from non-polar to polar. Separation in GC is mainly based on two different mechanisms. On non-polar stationary phases such as polydimethylsiloxane (PDMS), the separation takes place predominantly as a function of the differences in vapor pressure (and therefore boiling point) between of analytes. The separation on polar stationary phases is based on selective interaction between analytes and the phase, for instance hydrogen bonding and dipole interaction between polar analytes (e.g. alcohols, aldehydes) with polyethylene glycol type phases (WAX or free fatty acid phase, FFAP); however, boiling point separation also plays a role when using these columns. Semi-polar phases typically consist of mixtures of PDMS and polydiphenylsiloxane and/or cyanopropyl groups, providing mixed retention mechanisms. Usually columns are selected according to the “like-dissolves-like” principle. Lower polarity phases are, though, most commonly used for non-polar and semi-polar volatiles as they show better peak shapes and have higher temperature stability. Published retention indices (see below) of target analytes can also be very helpful when selecting a phase for a specific application (*2*).

The inner diameter has an impact on the efficiency, speed, and loading capacity of a capillary column. According to Golay’s work (3), both the efficiency and the optimal carrier gas velocity (of open tubular columns) are inversely related to the column diameter. Note that the carrier gas pressure increases as the internal diameter decreases. Columns with larger diameters are normally coated with a thicker film. Increased film thickness, in turn, provides larger capacity of the column, thus preventing overloading; this however increases separation time. For samples containing compounds present at widely varying concentrations the possibility of co-elution as a result of broad, overloaded peaks with compounds of interest is reduced using a thicker film column. As both the film thickness and inner column diameter affect the elution temperature, the phase ratio (Equation 1), which

combines the two factors, is often used to evaluate the suitability of a column for an application.

Equation 1

$$\beta = \frac{d}{4d_f}$$

Where β is the phase ratio, d the internal diameter and d_f the film thickness. The phase ratio gives a dimensionless value characterizing column internal diameter and film thickness combinations. Columns with a small phase ratio (thick film) are better suited for the analysis of very volatile compounds, whereas thin film columns with a larger phase ratio are superior for high molecular weight compounds (*2*).

The choice of column depends to a large extent on the complexity of the sample. For very complex matrices containing many compounds, such as petroleum or wine, longer columns of up to 60 - 100 m are preferred. The analysis time increases with increasing column length. On the other hand, columns with very small inner diameters of ~ 0.1 mm used in fast GC provide a fast separation and are only 10 to 20 m long. Note that these high performance columns provide the same efficiency as longer, wider bore columns allowing much faster analyses, although they have low capacity. The gas pressure, however, is the limiting factor for the length of a GC column, as it is directly proportional to length and inversely to the internal diameter.

Retention indices present a standardized system to express gas chromatographic retention data, which can aid in identification by comparison with linear hydrocarbon standards. A series of closely related standard substances, most commonly a series of *n*-alkanes, is used to describe the retention behavior of the compounds of interest on a specific stationary phase. The retention index (RI) is interpolated by relating the retention time of the compound of interest to the retention time of two standards (*n*-alkanes) eluting before and after this compound. Experimental RI values can be compared to values reported in the literature and available in RI-databases. The calculation of retention indices for isothermal separation is done according to Kováts (Equation 2) and for analyses carried out using a temperature programming according to Dool and Kratz (Equation 3). The retention indices of *n*-alkanes are by definition equal to 100 times their carbon number for any stationary phase and any given temperature. For instance octane (C_8) has a retention index of 800 (*5*).

Equation 2

$$I_S^{\text{st. phase}}(T) = 100[z+(\frac{\log X_S - \log X_z}{\log X_{(z+1)} - \log X_z})]$$

Where I is the isothermal retention index at temperature T, S is the compound of interest and st. phase is the stationary phase. Furthermore, X is the retention time for the compound S and the *n*-alkanes with z carbon atoms used for the calculation (*5*).

Equation 3

$$I^T = 100[z+(\frac{t_{Ri}^T - t_{Rz}^T}{t_{R(z+1)}^T - t_{Rz}^T})]$$

Where, I^T is the programmed-temperature retention index, also called linear retention index (LRI) and t_R the retention time for the compound of interest i and the *n*-alkanes with z carbon atoms (*5*).

2.1.2 GC injectors

The injector serves to introduce gaseous or liquid samples into the analytical column. The introduction of liquid samples is often problematic for various reasons. First of all the injection of a sample into the injector must be fast to avoid band broadening. Second, evaporation should be instantaneous and not lead to analyte decomposition and compound discrimination. Furthermore, the evaporated sample must be introduced into the analytical column as a sharp band. Finally, the loading capacity of the column as well as the linear range of the detector should not be exceeded (overloading). Several different types of injectors for capillary GC have been developed over the last decades (*4*).

2.1.2.1 Split/splitless injection

The most widely used injector in capillary GC is the split/splitless injector. This is a vaporizing injector, where the sample is injected at high temperatures, causing instantaneous vaporization prior to its introduction into the analytical column. As the name suggests, the injector can be used in either the split mode or the splitless mode.

In capillary GC, as in every other binary partitioning system, the amounts of both phases limit the sample capacity. The low amount of stationary phase and the small free gas volume therefore result in very low sample capacities for capillary GC columns. To overcome the problem of overloading a certain amount of the sample can be discarded from the injector (split mode), provided that the analytes of interest are present at relatively high concentrations. The injected sample evaporates instantaneously and mixes with carrier gas before a certain ratio of this mixture is discarded via the split valve. The remaining sample-gas mixture is introduced into the column. The split ratio is regulated via the total gas flow through the injector and the column flow. Typical split ratios vary from 10:1 to 100:1.

For trace analysis, where high sensitivity is required, the injector can be operated in splitless mode. In this mode the split valve only closes shortly before the injection, and opens after the complete sample is introduced into the column (typically after 0.5 - 2 min). A normal injection volume of for instance 1 µL significantly exceeds the capacity of an ordinary capillary GC column (the vapor volume of 1 µL corresponds to ~ 40% of the typical column volume). To prevent the undesired phenomena associated with overloading, such as markedly broadened peaks and a large solvent peak, the utilization of focusing mechanisms is obligatory in splitless injection. The “solvent effect” entails the trapping of volatile analytes in a temporary liquid phase formed by the re-condensed solvent at the beginning of the column. The partitioning of the analytes in this solvent film results in narrower peaks, and, therefore, prevents peak broadening. In splitless injection the choice of the solvent as well as the initial temperature of the oven program must always be carefully considered to achieve this effect. As a rule of thumb the boiling point of the solvent should be ~ 20°C above the initial oven temperature and the polarity of the solvent similar to the polarity of the stationary phase. For semi-volatile analytes the solvent effect is less effective and thermal focusing, often together with stationary phase focusing, is required. This entails the use of a retention gap (an uncoated piece of capillary) prior to the column (*2, 4*).

2.1.2.2 The programmed-temperature vaporization injector

The operation principle of the programmed-temperature vaporization injector (PTV) is similar to the traditional split/splitless injector, with the exception that the temperature of the PTV can be more rigorously controlled, including the possibility of cooling the injector. Another characteristic of the PTV is the use of a liner with a significantly smaller diameter as compared to conventional split/splitless injector. This ensures rapid heat transfer during heating of the injector, however, the capacity of the liner is much smaller and overloading of the injector easily occurs. The PTV can be cooled with different agents such as liquid nitrogen or carbon dioxide. The PTV offers the possibility of solvent elimination by solvent

venting, especially beneficial for trace analysis. In solvent vent mode, the sample is injected into a cool injector, which allows the low-boiling solvent to evaporate. The evaporated solvent is vented via the split valve, while the semi- and less volatile analytes remain in the injector. Subsequently, the split valve is closed and the PTV is heated up ballisticaly for fast introduction of the analytes into the column. In this manner, the injection of large sample volumes (large volume injection, LVI) can also be realized (*2, 4*).

Due to the possibility of the application of very low temperatures (down to -150°C) the PTV is also ideally suitable as a cryogenic trap following thermal desorption or dynamic headspace sampling.

2.1.2.3 Cool on-column injection

Cool on-column injection is a technique of introducing a sample as a liquid directly onto a GC column. This approach eliminates sample discrimination and sample degradation, while providing extremely accurate results. As the compounds begin the chromatographic process at relatively low temperatures, cool on-column injection is very suitable for thermally labile components, since they are not exposed to thermal stress. Cool on-column injection has some drawbacks. Samples must be relatively clean, since they are injected directly on to the column. In addition, sample dilution is required to avoid overloading the column as real samples are usually too concentrated.

2.1.2.4 Thermal desorption

Thermal desorption is used to desorb analytes from trapping materials following sorptive or adsorptive sample extraction. Sorptive sample preparation techniques will be discussed more in detail below. The two thermal desorption devices used in this study (Thermal Desorption System; TDS, and Thermal Desorption Unit; TDU, Figure 1) are both manufactured by Gerstel (Mülheim an der Ruhr, Germany). These devices differ in their design, but the operating principle is the same. In both systems the sample or the sampling device is placed in a removable glass desorption tube, which is flushed with carrier gas at a constant flow (the desorption flow) at a programmed temperature (the desorption temperature). The thermally desorbed analytes are transferred to a pre-cooled PTV injector mounted underneath the TDU or TDS, where they are cryo-trapped. Liquid nitrogen is usually used to cool the PTV, since temperatures down to -150°C can be reached with this coolant. Following cryo-trapping, the PTV is rapidly heated to introduce the analytes into the analytical column. Both thermal desorption devices can be operated in split and/or splitless modes.

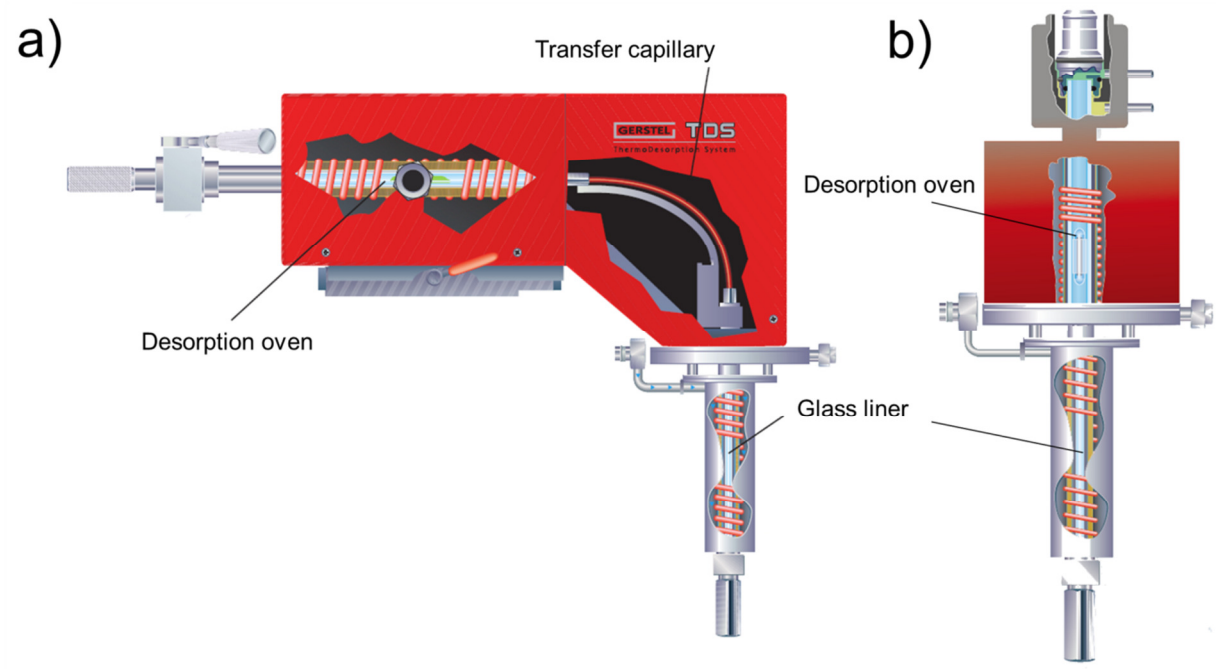

Figure 1: The two thermal desorption devices from Gerstel mounted on top of a PTV injector Cold Injection System 4 (CIS4) a) Thermal Desorption System (TDS) b) Thermal desorption Unit (TDU) (*6, 7*).

2.1.3 GC detectors

After separation the carrier gas and analytes elute from the column and pass through a detector. This device generates an electrical signal, which is either dependent on the concentration of the analyte in the carrier gas or the mass of analyte passing through the detector. In both cases the electrical signal is proportional to the amount of the analyte. The electrical signal is recorded by means of computer software, which is also used for further data processing and analysis. The choice of detector is highly dependent on the composition of the sample and the concentrations and physiochemical properties of the target analytes. The detectors used in this study are described in more detail below. Other detectors used for GC include the electron capture detector (ECD), nitrogen phosphorus detector (NPD) and atomic emission detector (AED).

2.1.3.1 The flame ionization detector

The flame ionization detector (FID) is the most commonly used detector. In the FID a hydrogen flame is used to ionize organic molecules in the traversing gas stream. As a negative polarizing voltage is applied between the jet tip where the flame is located and a

ring electrode, ions and electrons produced by destruction of organic analytes cause a current to flow in this gap. The amplification of this current results in an electrical signal. In the FID only compounds containing at least one hydrogen-carbon or carbon-carbon bond can be detected, whereas permanent gases give no response. High versatility, high sensitivity, low cost and robustness make the FID an important all-round, universal detector (*1*, *2*).

2.1.3.2 Mass spectrometry

The advances in mass spectrometry (MS) in the last two decades and the availability of inexpensive benchtop instruments have made GC hyphenated to MS (GC-MS) one of the most widespread techniques for the identification and quantification of organic compounds in complex matrices. In GC-MS, compounds eluting from the GC column in the gas phase are first ionized in the ion source of the mass spectrometer. The produced ions are guided through various lenses into the analyzer. The mass analyzer separates the ions according to their mass-to-charge (m/z) ratios. As almost all ions in GC-MS have a single charge, the m/z value also corresponds to the mass of an ion. The ions exiting the mass analyzer are detected by an electron multiplier. In the electron multiplier a cascade of electrons is generated resulting in an amplification of the signal (*1, 2*).

In GC-MS, the two most common ionization modes are electron impact ionization (EI) and chemical ionization (CI). The type of ionization and the relative energies of the produced ions define the degree of fragmentation of the ions. In EI, the gaseous analytes interact at low pressures with electrons accelerated through a 70 V electric field supplied from a filament. This process results in positively single-charged molecular ions. As EI is a very energetic process a considerable amount of the formed molecular ions undergo extensive fragmentation to produce positively singly-charged lower molecular mass fragments. This fragmentation takes place in less than one microsecond after the formation of the intact molecule ion. An ionization energy less than 15 eV is usually enough to ionize most organic molecules. The use of 70 eV has become the norm due to the requirement of comparing mass spectra obtained from different instruments, which allows the establishment and use of mass spectra libraries. In contrast to EI, CI is a less energetic ionization process, referred to as a “soft ionization” technique. In CI the ionization of molecules occurs indirectly via a reaction gas. The reaction gas, such as methane, is ionized by accelerated electrons from a filament, similar to EI. Following some intermediate ion/molecule reactions several ionized species of the reaction gas convert analyte molecules into ions through physical collision. Due to the fact that CI uses significantly less energy for the ionization of the analyte molecules, the obtained base peak (the most intense ion in the mass spectrum) in CI mass

spectra is often the molecular ion, and little or no fragmentation is observed. Considering that only 20 % of the EI mass spectra contained in the NIST08 Mass Spectral Database show a molecular ion peak, CI can be considered as a complementary technique to determine or confirm the molecular weight of a compound and for structure determination (*1*, *2*).

The three most common mass analyzers, the quadrupole (qMS), time-of-flight (TOF) and quadrupole ion-trap (QIT) analyzers, were used in this study and are discussed below.

The quadrupole mass filter consists of four electrical rod-shaped poles (electrodes), which are arranged so that two similar poles are placed across from each other, whereas opposing electrodes have the same potential. An oscillating electrical field is created by applying a negative direct current (dc) potential on one pair of opposing electrodes and a positive dc voltage on the other pair, while simultaneously applying a fixed radio frequency (rf) to all the electrodes. For a specific ratio of the rf amplitude relative to the dc amplitude, only ions with a specific m/z value will remain in the alternating field, whereas all other ions are diverted out of the mass filter. From a continuous beam of ions produced in the ionization chamber, only ions with this m/z ratio will be allowed through the quadrupole to be detected by the electron multiplier. Filtering of a wide range of m/z values, which is referred to as scan mode, is obtained by increasing (or decreasing) both the dc and rf amplitudes, while keeping the ratio between them constant. Depending on the scanned mass range a mass spectrum is obtained in ~ 0.1 seconds. A full mass spectrum allows the identification of compounds by comparison with databases of mass spectra, such as mass spectral libraries available from National Institute of Standards (NIST) or Wiley. However, the sensitivity in scan mode of conventional quadrupole mass spectrometers is often not sufficient for trace analysis, since the dwell time for a specific ion in the mass analyzer is very short. Enhanced sensitivity and selectivity can be achieved by selecting only a few ions to be analyzed (selected ion monitoring, SIM), as the same dwell time as for the full scan is then apportioned between only a few ions, resulting in longer dwell times for each ion. Although mass spectral information is lost, SIM mode is primarily used for trace-level analysis (i.e. where the identities of the target analytes are known) (*1*, *2*).

The quadrupole ion trap (QIT) mass analyzer is composed of a doughnut shaped ring electrode with two end-cap electrodes, which provide the ion entrance and the ion exit, respectively. After analytes are ionized by EI or CI, the formed ions enter the trap and are stored in an alternating electric field, which is created analogously to the electric field of the quadrupole, by applying dc potentials and fixed rf to the electrodes. The trapped ions circulate in between the electrodes in three dimensional concentric orbitals, where ions with higher m/z values are closer to the center. The amplitude of the fixed rf on the ring electrode defines the lowest m/z value kept in the trap. By increasing the amplitude of the rf, ions of one m/z value at a time are destabilized from their orbit and get attracted from the end-cap

electrodes. The destabilized ions exit the trap through one of the end-cap electrodes and are accelerated into the detector. A full range mass spectrum can be generated in this way. In contrast to quadrupole mass analyzers, detection sensitivity decreases only slightly with the number of selected ions when the QIT is operated in SIM mode. The possibility of using SIM mode with ion trap systems is the basis to perform tandem mass spectrometry (MS/MS) detection. In principle, MS/MS consists of three consecutive steps: selection of precursor ions, collision-induced dissociation and analysis of the product ions. In a QIT instrument all three steps take place in one location. MS/MS analyses performed in QIT instruments are therefore referred to as “tandem-in-time”. The analyte molecules are ionized and enter the ion trap. The selection of a precursor ion is performed by mass separation in SIM mode (MS^1; the remainder of the ions are ejected from the on trap). The pre-selected ion is subsequently fragmented by collision of the ion with neutral gas molecules (CID, collision induced dissociation; or CAD, collision activated decomposition). In QIT instruments helium is used exclusively as a collision gas. The kinetic energy of the precursor ion is converted into internal energy, which results in characteristic fragmentation of the precursor ion. A second mass separation (MS^2) for the analysis of the formed product ions is then carried out either in SIM or in scan mode. The combination of SIM-Scan (MS^1-MS^2) results in the product ion spectrum. This approach is used for the identification and confirmation of compounds and for structural determination. Alternatively, the combination of SIM-SIM provides the intensity for selected product ions and is used for highly selective and sensitive quantification of target compounds in complex matrices. Note that MS^1 can also be operated in scan mode (*1*, *2*).

The time-of-flight (TOF) mass analyzer is, due to its mode of operation and design, probably the simplest analyzer for mass spectrometry. Following the formation of ions in the ion source, an accelerated beam of ions is introduced into the ion modulator. A bundle of ions of all m/z values is orthogonally deflected into a field free flight tube by means of a pulsed electric field (kHz range). As the same kinetic energy is transferred to all ions, those with low m/z values travel faster than ions with high m/z values. Hence, ions are separated along the field free flight tube as a function of their velocity. Note that due to the mechanism of mass separation the TOF-MS can only be operated in scan mode. Most TOF-MS instruments are equipped with a reflectron. This “ion mirror”, which consists of an electric field, enhances the mass resolution by compensating for differences in kinetic energy of ions with the same m/z values. The advantages of the TOF-MS instruments include the possibility of performing accurate mass measurements (± 0.002 millimass units) or high data acquisition rates of up to 500 spectra per second at unit mass resolution. As a result TOF-MS is the only mass analyzer which provides sufficiently fast data acquisition rates (> 100 Hz) for fast GC and comprehensive two dimensional GC (GC×GC) (*1, 2*). Increased acquisition rate, however,

results in lowered sensitivity. For instance, an increase of the acquisition rate from 5 Hz to 50 Hz results in a 90 % decrease of ion abundance (*2*).

2.1.3.3 Sulfur chemiluminescence and nitrogen chemiluminescence detectors

The ozone-induced chemiluminescence based sulfur chemiluminescence detector (SCD) and nitrogen chemiluminescence detector (NCD) are unique and powerful detectors for the selective detection of sulfur and nitrogen containing species, respectively. The operation principles of both detectors are very similar. In both detectors compounds containing sulfur or nitrogen are converted in a first step into molecules capable of reacting with ozone to produce characteristic chemiluminescent emission. Subsequently chemiluminescence is induced and the emitted wavelength is detected (*8*). In the NCD. compounds eluting from the column react with oxygen at high temperature (~ 1000 °C). The products formed from this pyrolitic reaction are carbon dioxide (CO_2), water (H_2O), nitric oxide (NO), sulfur dioxide (SO_2) and other oxides (MO_x). Subsequently NO reacts with ozone (O_3), resulting in the formation of excited nitrogen dioxide (NO_2*). The decay of NO_2* to the ground state causes a near infrared chemiluminescence emission around 1200 nm, which is detected by a photomultiplier tube equipped with optical filters (Equation 4) (*8*, *9*).

Equation 4

$$NO + O_3 \rightarrow NO_2^* \rightarrow NO_2 + h\nu$$

In the SCD the eluting compounds are also oxidized at very high temperature, resulting in the same oxidation products as in the NCD. The formed SO_2 does not show chemiluminescence with ozone and therefore further reaction of the gases with hydrogen is necessary to produce sulfur chemiluminescent species (X-S) (Equation 5). The identity of these species remains unclear, although they are widely believed to include sulfur monoxide (SO). The intermediate product of the sulfur chemiluminescence species and ozone is excited sulfur dioxide (SO_2*). Analogously to the NCD, the excited species decay to the ground state resulting in emission of electromagnetic radiation of wavelengths ranging between 280 - 460 nm with a maximum around 360 nm (Equation 6). A photomultiplier tube equipped with optical filters is also used for detection (*8*).

Equation 5

$$SO_2 + H_2 \rightarrow X\text{-}S$$

Equation 6

$$X\text{-}S + O_3 \rightarrow SO_2^* \rightarrow SO_2 + h\nu$$

2.1.3.4 Flame photometric and pulsed-flame photometric detectors

The flame photometric detector (FPD) and the more recently developed pulsed flame photometric detector (PFPD), are two additional sulfur-selective GC detectors. In the FPD compounds in the GC column effluent are burned in a hydrogen/air flame, similar to the FID. This combustion triggers a chemiluminescence reaction during which sulfur containing compounds form excited disulfur molecules (S_2^*), which emit a characteristic band of radiation at 394 nm. As interfering background emission from hydrocarbons may occur, narrow band pass filters must be used, which limits the sensitivity of the FPD. The PFPD overcomes this drawback. The main difference between the PFPD and the FPD is the use of a pulsed flame in the former, which extinguishes and is reignited 3-4 times a second. A very low hydrogen flow is used in the PFPD, so that the ignited flame extinguishes by itself. The chemiluminescence produced by sulfur species occurs later (6-26 ms) than the emission caused by carbon and oxygen bonds (1-3 ms). Due to the fact that the combustion is cyclic and not constant in the PFPD, the earlier occurring interfering emissions of carbon and oxygen species can easily be filtered out by switching the photomultiplier off at the beginning of every cycle. In addition to sulfur containing species, both types of detectors can also be converted for the detection of compounds containing phosphorus and other elements (*1, 2 ,10, 11*).

2.2 Sample preparation

Sample preparation is a crucially important step prior to GC analyses. On the one hand enrichment of analytes is often necessary to compensate for the limited sample capacity of capillary columns, especially in trace analyses. On the other hand compounds occurring in high concentrations can cause overloading of the column and co-elution and must therefore be reduced. Another important aspect of sample preparation is the elimination of interfering matrix constituents such as water or co-eluting compounds and, in addition, the removal of non-volatile constituents which can contaminate the injector and the column. Universal detectors such as the FID or quadrupole MS demand higher selectivity of the sample preparation method compared to element-specific detectors (e.g. NCD, PFPD) or highly selective MS techniques such as MS/MS (e.g. QIT) or high resolution MS (e.g. TOF). The

different physiochemical properties of analytes and the matrix (e.g. volatility, polarity) as well as concentration ranges of the analytes of interest must be carefully considered when selecting a sample preparation technique for a particular analysis. The following discussion is focused on techniques which are used for the analysis of wine volatiles. Especially for the analysis of wine, the presence of non-volatile wine constituents can compromise GC analysis.

2.2.1 Liquid-liquid extraction

In liquid-liquid extraction (LLE) the aqueous sample is extracted with an organic solvent which is not water-miscible (or at least sparingly soluble in water). Analytes partition between the two phases according to their distribution coefficients (K_D's). Organic solvents used for the extraction of aqueous samples such as wine must be non-polar and must not dissociate in the aqueous phase or polymerize in the organic phase. Although LLE is still widely used for wine analysis, the technique is steadily being replaced by alternative methods due to several inherent disadvantages such as the large amounts of organic solvents required for quantitative extraction, uncomfortable solvent handling and the fact that establishment of equilibrium is often time-consuming. Several modifications of LLE have therefore been developed. Solid supported liquid-liquid extraction (SLE) improves recoveries and simplifies solvent handling during extraction by absorption of the sample from a solid support material and subsequent elution of analytes with an organic solvent (*12, 13*). Moreover, when the equilibrium between the two phases is characterized by large K_D's for the analytes of interest the amount of solvent can be significantly reduced. In this manner a more advantageous phase ratio is obtained, resulting in enhanced sensitivity. This approach is called micro liquid-liquid extraction (µLLE) (*14*, *15*). Higher temperatures and pressures may also be used in accelerated solvent extraction (ASE), resulting in decreased extraction time and improved extraction efficiency (*16*). Another approach is the exposure of the sample and solvent mixture to microwaves (microwave assisted solvent extraction, MASE) or ultrasound (ultrasonic assisted extraction, UAE) to increase recoveries. Especially for the extraction of volatiles in wine, alternative approaches to LLE have in recent years found increasing application (*17-19*).

2.2.2 Headspace sampling

When static headspace (HS) sampling is performed only volatile components in the gas phase above the aqueous sample are introduced into the GC. Highly volatile and semi-volatile compounds partition between the sample matrix and the headspace in a static

closed system (typically a closed headspace vial). Once equilibrium has been established, a certain volume of the headspace is introduced into the GC inlet by means of a gastight syringe or a sampling loop. HS injection is a very clean sample preparation method for GC analysis, since all non-volatile components remain in the sample matrix. Neither extraction nor a clean-up step is required, which prevents the loss of analytes. Better sensitivity can be achieved by affecting the partioning of analytes between the headspace and the aqueous sample matrix by increasing the extraction temperature and utilization of the salting out effect. A variation of this technique also based on the partioning of analytes into the headspace is dynamic headspace sampling. After agitation and purging of the sample with an inert gas, analytes are usually trapped using adsorbent or sorbent materials followed by thermal desorption. Dynamic headspace sampling results in much higher sensitivity at the cost of longer extraction times and method complexity.

2.2.3 Solid phase extraction

Solid phase extraction (SPE), due to its flexibility, has found widespread application as an alternative sample preparation method to LLE. The principle of SPE can be compared with liquid chromatography. Prior to sample introduction the sorbent material packed in a cartridge is conditioned with organic solvent. After the sample is introduced interfering compounds are rinsed from the cartridge, whereas the analytes of interest remain in/on the stationary phase. After the clean-up step the analytes of interest are eluted from the stationary phase with a strong solvent. The utilization of different separation mechanisms such as adsorption, partitioning, affinity or ion exchange and the use of organic solvents with different properties make this technique accessible for the analysis of a wide range of compounds. For the analysis of organic compounds C18 and styrene-divinyl benzene (SDVB) phases are most often used (*20*).

2.2.4 Sorptive or partially sorptive sample preparation techniques

Sorptive materials are mostly polymeric phases, which are above their glass transition points (T_G's) at the temperatures at which they are applied for extraction and therefore act as a non-miscible liquid phase. These materials are usually homogeneous and non-porous. The sorptive extraction process is understood as dissolution of compounds in the sorptive material, which can be compared to the partitioning process in liquid-liquid extraction. The fact that compounds do not temporarily bind with the sorptive material demarcates the sorption process from adsorption. Temperature plays an important role, as materials only act as sorbents above their T_G's. At lower temperatures these materials can act as adsorbents

with low surface areas. There are only a few polymers which show the characteristics of sorptive materials in the typical temperature range for sample preparation temperatures (0-30°C). Polydimethylsiloxane (PDMS) is the most widely used sorbent, as it does not only meet the sorption requirements, but also shows high inertness, excellent thermal stability (up to 320°C) and beneficial diffusion properties. Moreover, all PDMS degradation products contain silicone, which facilitates the differentiation of analytes of interest from degradation artifacts by means of siloxane fragments present in the mass spectrum. The affinity of analytes in aqueous matrix for PDMS can be estimated by their octanol-water partitioning coefficients ($K_{O/W}$), as these are proportional to the partitioning coefficient between PDMS and water ($K_{PDMS/W}$). The most important sorptive sample preparation techniques are open tubular traps (OTT), solid phase microextraction (SPME, which also sometimes combines sorption and adsorption materials, referred to as 'mixed phases') and stir bar sorptive extraction (SBSE). As the latter two techniques were used in this study, they are discussed in more detail below.

2.2.4.1 Solid phase microextraction

Solid phase microextraction (SPME) was developed by Arthur and Pawliszyn (*21*) in 1990, as a solvent free sample preparation method for aqueous sample matrices. In SPME a 1 cm long, 100 µm thick, fused silica fiber coated with a sorbent is either immersed in the sample or exposed to the headspace above the sample. During the sampling procedure analytes partition between the fiber coating and the sample resulting in an enrichment of analytes in the fiber coating. After sampling, the fiber is removed from the sample (ideally after equilibrium is reached) and the analytes are thermally desorbed in a conventional split/splitless GC injector at elevated temperatures. Several parameters such as the type of coating, extraction time and temperature, addition of salt (alteration of ionic strength), volume of the sample and the volume of the headspace affect the extraction of analytes. Since the introduction of SPME a range of different fiber coatings varying from PDMS to polar or mixed coatings has become commercially available. Most polymers used for SPME coatings are silicones related to GC column stationary phases. Other phases such as copolymers and physical mixtures of PDMS with inorganic adsorbents are also used, exploiting the respective advantages of the sorption mechanism and adsorption. However, using these mixed coatings will also inadvertently exhibit their respective disadvantages as well. One major disadvantage of SPME is the limited amount of sorbent on the fiber (0.5 µL) leading to a lack of sensitivity, especially for trace level analyses.

2.2.4.2 Stir bar sorptive extraction

Stir bar sorptive extraction (SBSE) was developed by Baltussen and co-workers (*22*) in 1999 to overcome the abovementioned drawback of the inherent limited sensitivity of SPME. In SBSE a magnetic stir bar of 1 - 2 cm in length is coated with a PDMS phase with a thickness of 0.5 - 1.0 mm. These stir bars are marketed by the company Gerstel under the name Twister®. For extraction the aqueous sample is usually transferred into a headspace vial and the Twister is immersed in the sample, but it can also be exposed to the headspace by means of an open glass adapter insert (headspace sorptive extraction, HSSE).

When using a PDMS phase in immersion mode the extraction of analytes in both SPME and SBSE can be described as follows, by assuming that the approximate partitioning coefficients between PDMS and water ($K_{PDMS/W}$) are proportional to the octanol-water partitioning coefficients ($K_{O/W}$):

Equation 7

$$K_{O/W} \approx K_{PDMS/W} = \frac{C_{SBSE}}{C_W} = \frac{m_{SBSE}}{m_W} \times \frac{V_W}{V_{SBSE}}$$

Where the analyte concentrations in the PDMS and in the water phase are C_{SBSE} and C_W, respectively, the mass of analyte in the PDMS and the water phase are m_{SBSE} and m_W, respectively, and the volume of PDMS and the water phases are V_{SBSE} and V_W, respectively. If the term V_W / V_{SBSE} is replaced by the phase ratio β, the equation can be presented as:

Equation 8

$$\frac{K_{O/W}}{\beta} = \frac{m_{SBSE}}{m_W} = \frac{m_{SBSE}}{m_0 - m_{SBSE}}$$

Here the total mass of analyte in the sample prior to extraction is m_0. To obtain the extraction efficiency (recovery), Equation 8 must be transformed:

Equation 9

$$\frac{m_{SBSE}}{m_0} = \frac{\frac{K_{O/W}}{\beta}}{1 + \frac{K_{O/W}}{\beta}}$$

This equation clearly shows that the recovery of an analyte is only a function of its octanol-water partitioning coefficient ($K_{O/W}$) and the phase ratio (ß). A recovery of 50% would therefore be obtained when $K_{O/W}$/ß = 1. Extraction thus can be assumed as quantitative at $K_{O/W}$/ß values higher than 5.

The increased sensitivity of SBSE compared to SPME is therefore a result of the increased amount of sorptive phase (decreased phase ratio). This can be demonstrated by using Equation 9 to compare the theoretical recoveries obtained by SPME and SBSE as a function of analyte $K_{O/W}$ value (Figure 2). In the case of SPME, for a 10 mL sample and a maximum phase volume of 0.5 µL PDMS (100 µm film thickness) the phase ratio would be 20000. With such a high phase ratio quantitative extraction ($K_{O/W}$/ß = 5) is only obtained with analytes with very high $K_{O/W}$ values above 100000. In contrast to SPME, a stir bar coated with 100 µL PDMS corresponds to a phase ratio of 100 for the extraction of a 10 mL sample. Quantitative extraction into the PDMS coating is then already reached for analytes with $K_{O/W}$ values higher than 500. Additionally, compared to SPME, sensitivity is increased for analytes with $K_{O/W}$ value as low as 10.

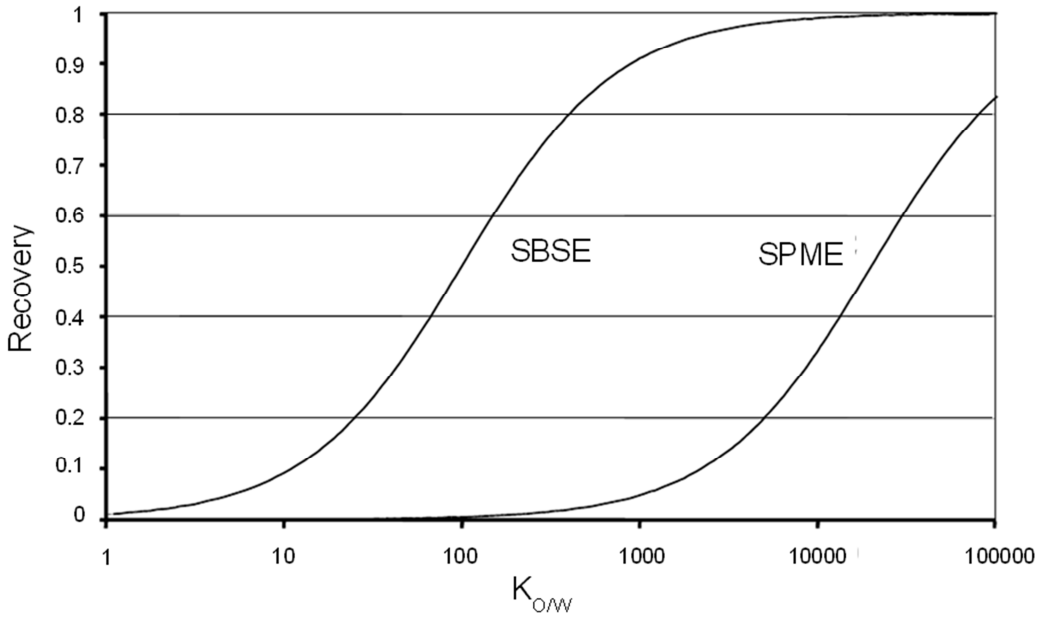

Figure 2: Comparison of theoretical recoveries of analytes as a function of their octanol-water partitioning coefficients for SBSE (100 µL PDMS) and SPME (0.5 µL PDMS) extraction using a 10 mL water sample (Adapted from (*22*)).

During the last decade efforts have been made to developed new stationary phases for SBSE to overcome its main disadvantage, namely that PDMS is rather non-polar and extraction efficiency is lower for polar analytes. However, only one alternative phase is currently commercially available. This phase consists of a PDMS/ethylene glycol (PDMS/EG) copolymer (EG-Silicone Twister) and was only recently introduced commercially.

2.3 Multidimensional gas chromatography

The term multidimensional chromatography describes the combination of two or more different separation mechanisms. In GC this is achieved by the coupling of two columns with different stationary phases, where a distinction is made between heart-cutting two-dimensional gas chromatography (GC-GC) and comprehensive two-dimensional gas chromatography (GC×GC). In heart-cutting GC only the fractions of interest from the first dimension column are transferred to the second dimension column, whereas in GC×GC the complete sample is analyzed in both dimensions by means of modulation.

By coupling two columns with different separation mechanisms the selectivity of multidimensional systems is enormously enhanced compared to one dimensional GC. The

importance of selectivity in terms of the chromatographic resolution (R_S) of a one dimensional system is illustrated in Equation 10.

Equation 10

$$R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_2}{1+k_2}$$

Where R_S is the chromatographic resolution of two adjacent peaks, N the number of theoretical plates of the column, α the selectivity of the chromatographic system for the two peaks and k_2 the retention factor of the second peak.

Equation 10 illustrates that for a one dimensional system extensive optimization of all factors influencing N and k_2 (column length, phase ratio, elution temperature and carrier gas velocity), is not as effective as the tuning of α. In one dimensional gas chromatography selectivity is largely determined by the choice of stationary phase (and the detection technique used). Co-elution of compounds is especially problematic for the analysis of complex samples containing a large number of components when a single separation mechanism is employed.

The need for a very large separation efficiency for the analysis of complex samples was illustrated by Davis and Giddings (*23*), who developed a statistical model of peak overlap. They used the overall peak capacity (n) of a chromatographic system as a measure of its separation efficiency. The peak capacity describes the maximum number of well resolved peaks, which could theoretically fit next to each other into the available separation space (*24*) and can be calculated according to Equation 11 (*25*).

Equation 11

$$n = 1 + \frac{\sqrt{N}}{4} \ln \frac{t_n}{t_0}$$

Where n is the peak capacity, N the number of theoretical plates, t_n the retention time of the last eluted compound and t_0 the void time.

The theoretical peak capacity is never reached in a practical separation, since peaks are randomly distributed over the chromatogram. As a logical consequence this fact is much more pronounced the more complex a sample. The peak capacity of a chromatographic

system must therefore be much higher than the number of randomly distributed peaks in order to decrease the degree of overlapping. Typical theoretical peak capacities of one dimensional GC separations are between 500-1000. This implies that practically only ~ 150 randomly distributed compounds can be separated by one dimensional GC.

In heart-cutting GC the overall peak capacity is the sum of the peak capacities of the first and second dimension, as illustrated in Figure 3 for two hypothetical separations with a peak capacity of 26 and 6 in the first dimension and second dimension, respectively.

In GC×GC the significantly higher separation efficiency is based on the assumption that the peak capacity of the GC×GC ($n_{GC\times GC}$) system is equal to the product of its first and second dimension peak capacities (n_{1D}, n_{2D}) as illustrated by Equation 12 (*26*) and Figure 3.

Equation 12

$$n_{GC\times GC} = n_{1D} \times n_{2D}$$

The following sample calculation illustrates the vast gain in separation efficiency in GC×GC: Supposing a typical peak capacity in the first dimension of 1000, and in the second dimension of 30, this would result in an overall peak capacity of the GC×GC system of 30000 (*2*). It should be noted that, similar to one dimensional peak capacity, the attainment of practical peak capacities according to Equation 12 is in practice rarely, if ever, achieved. This is due to the requirements of uncorrelated separation in the two dimensions and sufficient sampling rates of first dimension peaks, which are not always met (see below).

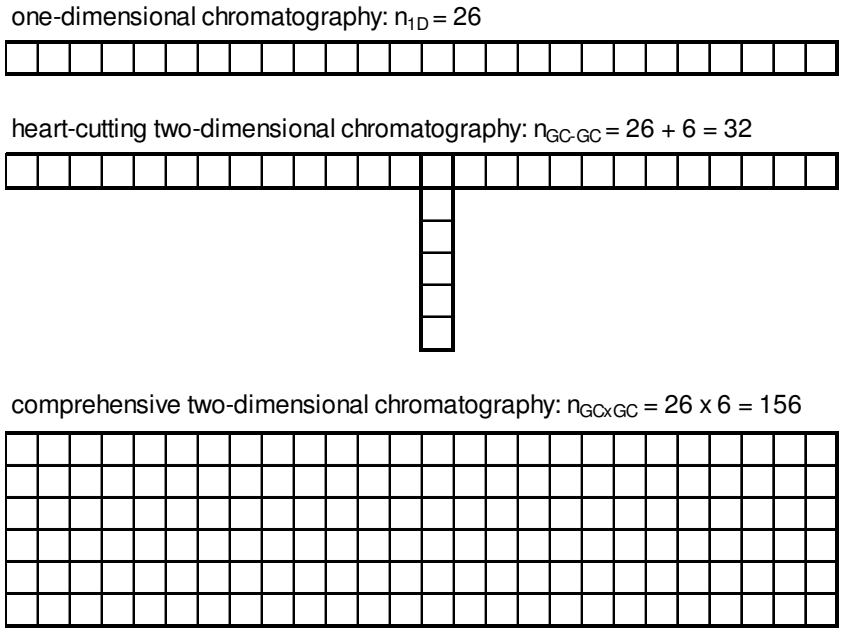

Figure 3: Schematic comparison of peak capacities in one-dimensional chromatography, heart-cutting and comprehensive two-dimensional chromatography for a first dimension peak capacity of 26 and second dimension peak capacity of 6 (adapted from (*2*)).

2.3.1 Heart-cutting two dimensional gas chromatography

Heart-cutting GC focuses on target compounds in a poorly resolved region of interest in the first dimension chromatogram. The compounds eluting in the section of interest from the first dimension column are transferred via a valveless flow switching device into the second dimension column. To observe the first dimension separation a small split is taken from the flow switching device to a monitoring detector, usually a universal FID. The valveless switching device is typically based on pneumatic pressure balancing. In the switching device the eluate from the first dimension column is directed either into the second dimension column or vented to waste by using programmed carrier gas flows (*27*, *28*). An example of such a device, the MultiColumnSwitching-System (MCS) from Gerstel, uses a counter flow to redirect the first dimension eluate to waste. Without this counter flow the first dimension eluate is passed on into the second dimension column (Figure 4) (*29*). Nowadays gas pressures are regulated using electronic pneumatic control (EPC). The EPC system is controlled by software integrated into GC software. The transferred compounds can be refocused using a cold trap at the beginning of the second dimension column. Alternatively, refocusing can also be obtained by the use of thick film columns or PLOT columns in the second dimension. The choice of column dimensions is independent from the heart-cutting procedure and should be optimized with respect to the application. The phase polarity of the

two columns should, however, be different to meet the requirement of orthogonality to effectively exploit the benefits of heart-cutting GC (*2, 29, 30*)

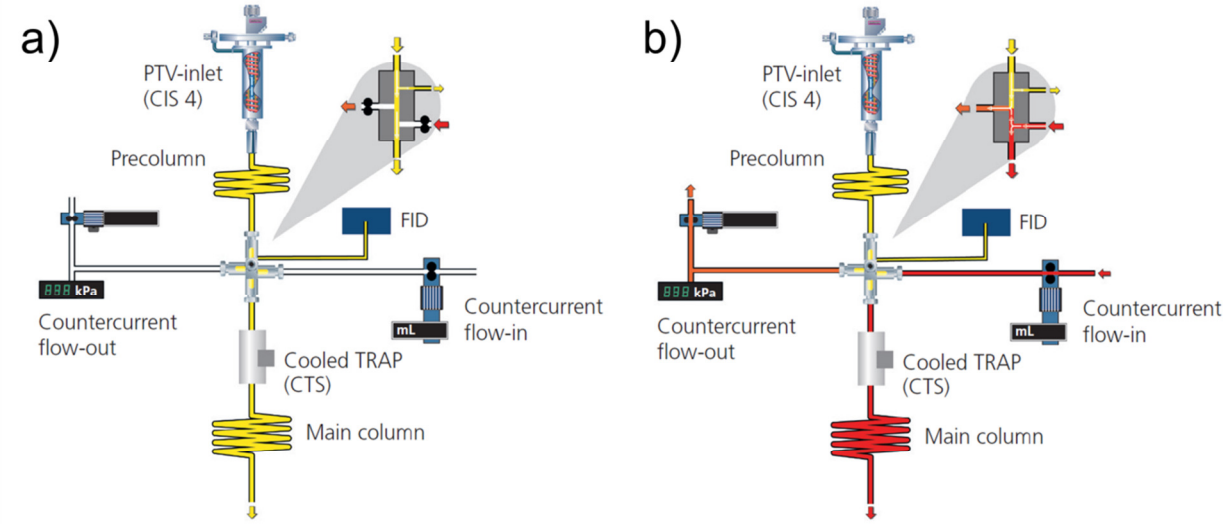

Figure 4: Scheme of the operation principle of the MultiColumnSwitching-System (MCS) from Gerstel (Cold injection system, CIS; cryogenic trapping system, CTS). a) The eluate of the first dimension column is directed into the second dimension column; counter current flow off. b) The eluate of the first dimension column is vented to waste; counter current flow on (adapted from (*29*)).

2.3.2 Comprehensive two-dimensional gas chromatography

2.3.2.1 Principles of comprehensive two-dimensional gas chromatorgraphy

Contrary to heart-cutting GC, in comprehensive two-dimensional gas chromatorgraphy (GC×GC) not only a single fraction of the first dimension separation is introduced into the second dimension column, but the entire sample. In principle GC×GC involves the combination of a conventional GC analysis in the first dimension with fast GC separation in the second dimension, with the two columns being connected by a modulator. The modulator is the “heart” of the GC×GC system, where the effluent of the first dimension column is frequently trapped and re-injected into the second dimension column. In this way, the whole first dimension separation is “cut” into consecutive second dimension chromatogram slices, resulting in a three-dimensional chromatogram, which is usually presented as a contour plot (Figure 5). The duration of one cycle of this procedure is called the modulation period.

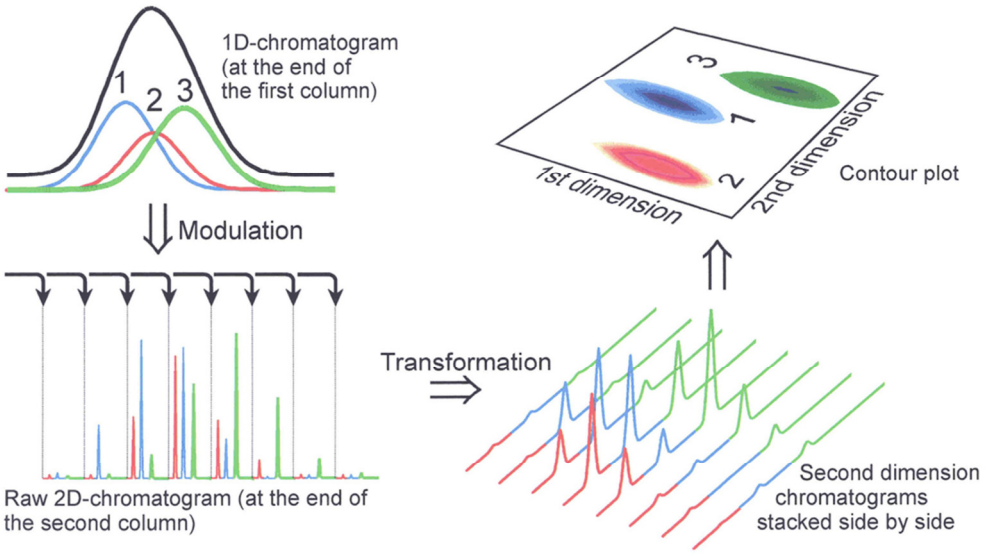

Figure 5: Generation and visualization of a GC×GC chromatogram (adapted and modified from (*31*)).

To exploit maximally the second dimension separation space in GC×GC, the separation mechanisms of the two columns should be uncorrelated (independent), or in other words orthogonal (*32*). Most commonly an apolar×polar column combination is used (referred to as a normal column configuration), but the use of other column sets such as polar×apolar (reversed column configuration) (*33, 34*), chiral×polar (*35*), phosphor ionic×apolar (*36*) or apolar×liquid crystalline (*37*) have also been reported.

Furthermore, it is essential to preserve the separation obtained in the first dimension (*32*). The preservation of the first dimension separation depends on the number of modulations per first dimensional peak. To maintain well resolved first dimension peaks, each first dimension peak should be sampled at least three to four times (*38*). As typical modulation periods in GC×GC vary from 3 - 8 seconds, the widths of first dimension peaks are supposed to be between 15 - 25 seconds. It is apparent that not every first dimension peak fits this criterion. Narrower first dimension peaks therefore often undergo only 1 or 2 modulations, leading to convergence with neighboring bands in the modulator. This is acceptable only as long as the overall quality of the separation is still adequate (*39*). If a too short modulation period is used, peaks with high affinity to the second dimension phase might not elute within their modulation cycle, but in the next one. This phenomenon is called “wraparound”. Therefore, a compromise between sufficient sampling of first dimension peaks and avoiding wraparound must be reached when modulator settings are chosen. For the first dimension separation usually a longer column of 15 - 30 m length, 0.25 - 0.32 mm internal diameter and 0.25 - 0.53 µm film thickness is used, whereas a very short column of 0.5 - 2 m, 0.05 - 0.2 mm internal diameter and 0.05 - 0.2 µm film thickness provides a very fast

separation in the second dimension. This is required to complete the second dimension separation during the modulation period and to avoid wraparound.

Besides the enhanced separation efficiency, GC×GC provides further advantages. Contour plots of GC×GC chromatograms often display structural retention patterns of related compounds, which allow group type identification. For instance, peaks of homologous series typically form lines in the GC×GC contour plots (*33, 40*). Structural retention patterns are especially useful if no standard compounds and/or library reference spectra are available, or when mass spectra for different compounds are very similar (e.g. terpenes) (*39*).

Another advantage of GC×GC is the improved sensitivity compared to one dimensional GC. The increased signal-to-noise ratios in GC×GC are a result of decreased peak width caused by re-focusing of analytes in the modulator and very fast second dimension analyses (*41*). The trapping of the effluent from the first dimension column in the modulator results in refocusing of the analytes by means of the combination of the stationary phase and cryo-trapping prior to reinjection into the second dimension column. This focusing step 'resets' band broadening obtained in the first dimension column. Therefore, refocusing in the modulator leads to a substantial increase in signal-to-noise ratios due to decreased peak width, and therefore to increased sensitivity for trace analyses. Peak widths obtained in the second dimension are typically between 100 – 500 ms. It is problematic to derive universal values for the degree of signal-to-noise enhancement in GC×GC; this depends on the modulation technique, gas flows, temperature programming conditions and secondary column characteristics such as length, diameter and film thickness. However, typical sensitivity improvement of 25 – 50× may be achieved (*39, 42, 43*).

The very narrow peak width of the second dimension separation requires fast detectors, small internal volumes and high acquisition rates. Good peak resolution requires at least ten data points per peak. Considering a peak width of 200 ms, the required acquisition rate is at least 50 Hz (data points per second). A slower acquisition will lead to incorrect peak reconstruction. Due to their high acquisition rates the two most common detectors for GC×GC are the FID and TOF-MS. The normal acquisition range of the FID is 50-200 Hz (*31, 33, 41*). Very high acquisition ranges up to 20 kHz has been reported for a modified FID, making this detector ideally suited for high speed GC (*44*). Most mass spectrometers have low acquisition rates in full scan mode and are therefore not suitable for GC×GC. An exception is TOF-MS, which provides acquisition rates up to 500 Hz (*45*). Other detectors have been used in combination with GC×GC include the micro electron-caption detector (μECD) (*46*), nitrogen phosphorous detector (NPD) (*47*), atomic emission detector (AED) (*48*) and sulfur chemiluminescence detector (SCD) (*49, 50*).

2.3.2.2 Modulation

Since the introduction of GC×GC 20 years ago (*51*) several types of modulators have been developed. In the first generation of thermal modulators, compounds were trapped in the stationary phase of a thick film second dimension column. Sequentially arranged heating spots produced by an electrical current passing through the resistive metallic coating on the capillary column triggers partitioning of the compounds back into the mobile phase (*51*). Similar devices have been developed by other groups (*52, 53*). In general thermal modulators show limitations for the analyses of very volatile compounds. The first commercially available thermal modulator was the “sweeper”. It consists of a movable heating device, which rotates repetitively back and forth over a piece of thick film capillary column, “sweeping” compounds focused in the thick film further into the second dimension column (Figure 6a). In addition to the use of moving parts, the restricted application range is the main disadvantage of this modulator (*54, 55*).

The introduction of cryogenic modulators marked a watershed in GC×GC research. Marriott and Kinghorn developed the first cryogenic modulator, the longitudinal modulating cryogenic system (LMCS), in 1997 (*56*). In the LMCS analytes are trapped using liquid carbon dioxide at the beginning of the secondary column when the trap is in the downstream position (position T in Figure 6b). Trapped and focused analytes are re-mobilized by moving the trap into upstream position (position R in Figure 6b), so that the region containing the analytes is heated by the oven (*57*). To initiate the re-evaporation process the cryogenic modulator only needs to be heated to oven temperature (Figure 6b). The disadvantages of this modulator include the use of moving parts and insufficient trapping of more volatile compounds due to the use of liquid carbon dioxide, which only provides trapping temperatures down to approximately - 50 °C.

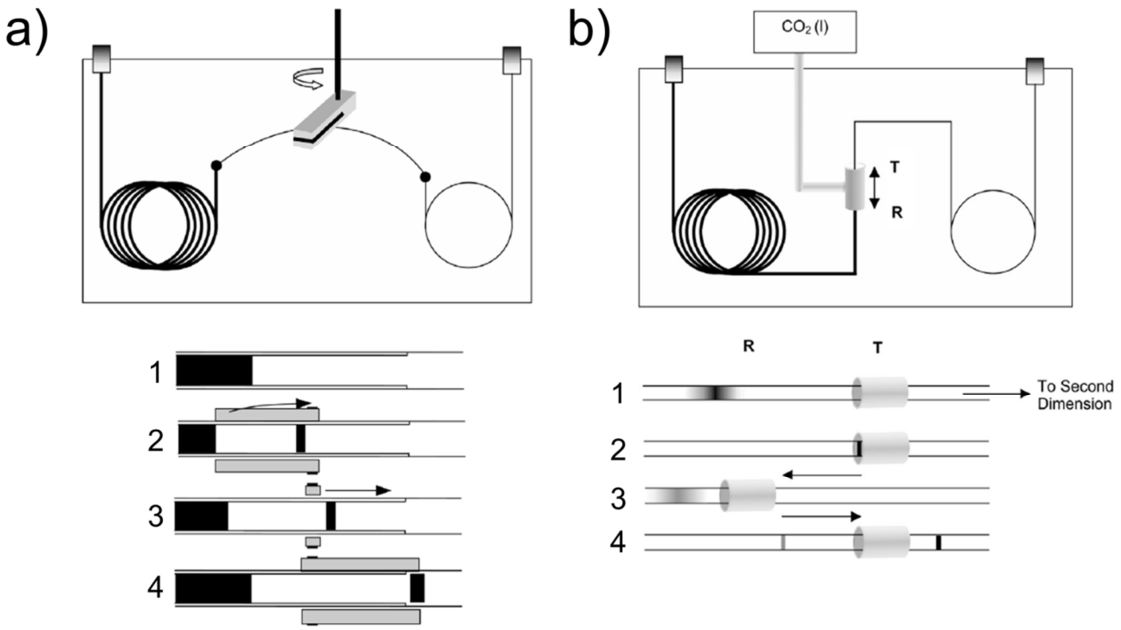

Figure 6: a) Scheme of the operation principle of the "sweeper": (1) A band elutes from the first dimension column and is focused in the thick film of the trapping capillary (2)&(3) the focused band is "sweeped" through the thick film capillary (4) the band is introduced into the second dimension column. b) Scheme of the operation principle of the LMCS: (1) The modulator is in trap position T when the analyte band enters the modulator (2) the analyte band is cryo-trapped and focused and (3) the trap moves to release position R while the analyte band is released into the second dimension column (4) the trap moves back to position T (adapted and modified from (*41*)).

The latest state-of-the-art modulators are cryogenic jet-based. Several different single jet or dual jet modulators have been designed. In all of them the escaping coolant gas from the cryojet produces a cold spot on either the first dimension or the second dimension column in which analytes are trapped. Re-mobilization of compounds through fast heating is achieved either by the use of a hot jet or simply by the oven temperature (Figure 7). The design and construction of jet-based modulators using carbon dioxide as coolant are simpler than of those using liquid nitrogen, as liquid carbon dioxide can easily be produced at room temperature under sufficient pressure. However, the effective focusing of highly volatile compounds requires the utilization of colder trapping temperatures than those obtainable with carbon dioxide. Therefore, liquid nitrogen is still often used as a cryogenic coolant (*58*). Effective modulation is achieved when the cold spots are sufficiently cold to cryo-focus compounds, yet not too cold so that compounds can be rapidly and completely evaporated at the oven or hot jet temperature. The optimal temperature therefore depends on the analytes of interest. Exceeding of the optimal modulator temperature results in tailing peaks, whereas

lower temperatures can cause peak broadening and loss of first and second dimension resolution.

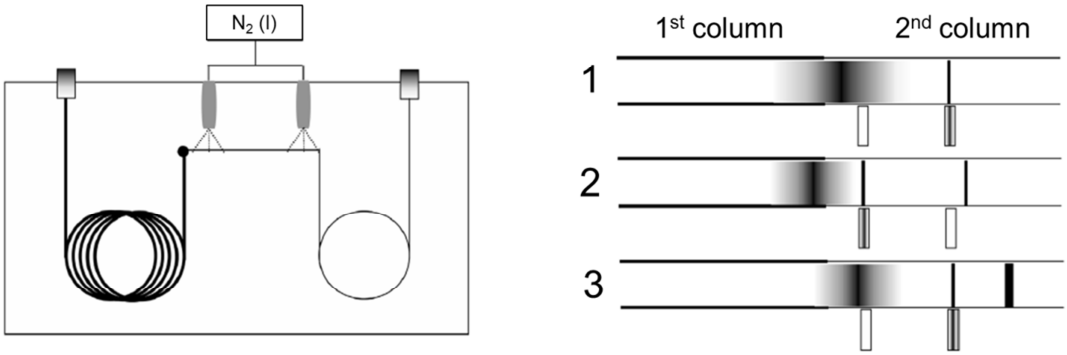

Figure 7: Scheme of the operation principle of the dual cryojet modulator. (1) An analyte band is trapped by the second jet (2) the second jet is turned off to release the band into the second dimension column, while the first jet is turned on to trap a next band (3) the first jet is turned off again to pass the band on to the second jet, which is turned on for sequential trapping. (adapted and modified from (*41*))

Valve based modulators are an alternative to the cryogenic approach. These include the use of multi-port valves and a sample loop to control sampling and re-injection of the first column effluent. In the first valve-based modulator set-ups, a certain amount of the first column effluent was vented to atmosphere when the previous sampled amount was introduced into the second dimension column by flushing the sample loop with a very high gas flow, so that the trapped analytes were injected as a narrow band into the second dimension column (*59, 60*). However, newer designs (differential flow modulation) made the modulation of the whole first dimension eluent possible by using two sampling loops (*61, 62*) or a stop-flow approach (*63*). To ensure fast flushing of the sample loops a higher flow rate for the second dimension column is used. A primary to secondary flow ratio of 1:20 is often used, so that the whole primary column eluate sampled in 1 second can be introduced as a narrow pulse of 50 milliseconds into the second dimension column (*64*). This flow ratio corresponds to very high flow rates of 20 – 30 mL/min in the second dimension column, which translates to very high velocities between 400 – 600 cm/s. Hydrogen is the best carrier gas to use here due to its low viscosity and higher optimal velocity compared to helium and nitrogen (*44, 65*). Differential flow modulation overcomes the use of large amounts of expensive cryogens such as liquid nitrogen and the limited trapping of high-volatility species associated with cryogenic modulators. The disadvantages of this technique are the requirement of hydrogen as carrier gas, and limited resolution in the second dimension resulting from high carrier gas velocities, which are far above optimal values (*65*). Regarding the extremely high flow rates in the

second dimension a more powerful turbo pump must be considered when mass spectrometric detection is used.

It is important to mention that there is no generic set-up regarding the column configuration and modulator to be used for a given sample. The choice is highly dependent on the specific application. For instance modulators using carbon dioxide might cause breakthrough of highly volatile compounds. On the other hand, wax-type phases limit applications in terms of maximum oven temperatures, and are therefore not suitable for the analysis of high-boiling compounds. Furthermore, cryogenic modulators are less suitable for field analysis, as the instrumental set-up is rather complex. A valve-based modulator would here be the better choice (*41*).

2.4 Gas chromatography in wine analysis

Besides water and ethanol, which are the main constituents of wine, it also contains a large number of other organic and inorganic compounds. The wine aroma is a result of perception of volatile wine constituents, which are detected by the human nose. Therefore aroma and flavor are essentially influenced by the composition of volatile compounds in wine. Gas chromatography is the method of choice for the analysis of volatiles and has therefore mostly been used for wine analysis.

Nowadays capillary columns are almost exclusively used due to their high separation efficiency. Different types of stationary phase coatings are used for the analysis of wine volatiles. Usually more polar phases such as Polyethylene glycol or modified PEG (WAX) (*66-70*) are prefered. However, other phases such as PDMS (*18*, *68*) phases or phases for special applications such as enantio-selective (cyclodextrin based) phases have also been used (*71*).

Although the analysis of wine aroma using direct injection into a PTV has been reported (*72*) it is very rarely done and in most cases neither practical nor feasible. When analyzing wine volatiles interfering matrix constituents such as non-volatiles and water must be considered. Often enrichment of target analytes is necessary, especially when they are present in low concentrations. The extraction of compounds is therefore essential prior to GC analysis. The properties of the target compounds determine the sample preparation procedure.

Aroma compounds present in higher concentrations in wine are often loosely referred to as ‘major volatiles’. These include esters, fatty acids, alcohols, and some compounds belonging to other chemical classes. These volatiles contribute to the base of the aroma profile and are present in almost every wine, albeit at different concentration levels. Several different sample preparation techniques have been used for the analysis of major volatiles; these include

mainly LLE and SPME, although other methods such as SPE and SBSE have also been used. It should be pointed out that using most of these methods some minor compounds are also detected during the analyses. LLE is, due to its simplicity, the most widely used extraction technique for major wine volatiles. The choice of solvent for LLE is critically important. Most often diethyl ether (*73, 74*), dichloromethane (*75-77*) and Freon (*66*, *78*) or mixtures of solvents such as dichloromethane/pentane (*79*) are used.

The trace analysis of minor volatiles is not straightforward, especially if the analytes of interest are polar or unstable. Sample preparation techniques for minor compounds must be suitable for removal of interfering matrix components (clean-up) and enrichment of the target analytes. Therefore SPE is a preferred method for the extraction of minor volatiles. High selectivity can be achieved by optimization of loading, washing and elution steps. For the selective extraction of wine constituents the following phases are most often used, depending on the chemical properties of the target analytes: reversed-phase C18 (*80*), Lichrolute EN (*81*, *82*) and styrene divinylbenzene (*83*).

The use of environmentally hazardous solvents is the main drawback of LLE and SPE, especially in the case of the greenhouse gas, Freon. Therefore solvent free techniques are gaining in popularity. SPME is a solvent free and fully automatable alternative to LLE and SPE. Since its introduction in 1990 (*21*) SPME has also extensively been used for the analysis of wine volatiles. A large selection of different fiber coatings with different characteristics offers variability in selectivity. The following fibers have been reported for the analysis of wine: PDMS (*19*, *84, 85*), carboxen/PDMS (CAR/PDMS) (*86*), PDMS/Divinylbenzene (PDMS/DVB) (*18*), DVB/CAR/PDMS (*18*), polyethyleneglycol/DVB (PEG/DVB) (*87*) and polyacrylate (PA) (*88*, *89*). SBSE is also suitable for the analysis of wine volatiles (*17, 90*, *91*). SBSE shows significant increase in sensitivity compared to SPME due to the higher phase volume. The improved sensitivity of SBSE makes this technique also suitable for the analysis of trace compounds in wine (*85*, *92*).

As GC×GC is still a young technique it has not been extensively used for the analysis of wine volatiles. High operating costs due to the use of cryogenics and the necessity of an expensive fast scanning TOF-MS for hyphenation with GC×GC are limiting factors for further application of this technique. GC×GC has been applied for qualitative characterization of wine volatile profiles (*93-95*) and quantitative screening approaches, such as the investigation of the effect of yeast strain, canopy management and field site on the volatile composition of Carbernet Sauvignon wines using HS-SPME-GC×GC-TOF-MS (*96*), the in-depth search for potential age markers of Madeira wine by HS-SPME-GC×GC-TOF-MS (*97*) and the investigation of the impact of micro oxygenation on the volatile composition of red wines using HS-SPME-GC×GC-qMS (*98*). Furthermore, GC×GC has been used to address the analysis of specific groups of compounds. Schmarr and co-workers used

HS-SPME with on-fiber derivatization in combination with GC×GC-qMS (*99*) for the analysis of wine aldehydes. 3-Alkyl-2-methoxypyrazines were analyzed by Ryan and co-workers using HS-SPME-GC×GC with both TOF-MS and NPD detection (*47*) and by Schmarr and co-workers (*100*) using GC×GC-qMS. In the latter study interfering matrix constituents were reported. HS-SPME-GC×GC-TOF-MS has also been used to study the ethyl carbamate content of Madeira wines (*101*). Pietra Torres and co-workers (*102*) applied GC×GC-TOF-MS to prove the identification of some tentatively identified wine volatiles in their study on the impact of MLF on the volatile composition of Trincadeira wines. In all of the above discussed approaches normal column configurations (apolar×polar) were used, except for the studies of Schmarr and co-worker (*98-100*) who used a reversed column configuration (polar×apolar).

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3

Wine volatiles

3.1 Introduction

Wine aroma is determined by the detection of a mixture of volatile wine constituents by the human nose. The most important quality criterion of wine is its the sensory characteristics, and in this regard the volatile composition plays an essential role. Therefore, dedicated information on the volatile constituents in wine is essential to the winemaker aiming to produce a product made, which fulfills consumer requirements in terms of sensory expectations (*1*, *2*).

The word *aroma* refers to the smell of a wine. Wine aroma is differentiated as follows: the *primary aromas* arising from the grapes (varietal). The *secondary aromas* derive from yeast and/or malolactic fermentation and the *tertiary aromas* are developed during maturation (barrel and bottle ageing). For the smell of a wine which has evolved during maturation in the bottle, the term bouquet is often used, which also expresses complexity. In contrast, wine *flavor* covers both the taste of a wine (sweetness, bitterness, acidity, saltiness, umami) and its aroma perception. The terms *aroma* and *flavor* are often incorrectly interchanged in popular usage (*2, 3*).

Natural products such as wine often contain hundreds of volatile compounds with different properties regarding their odor potentials. The sensory threshold is a very important characteristic of a volatile compound. A distinction is made between *perception threshold, recognition threshold* and *preference threshold* which are defined as follows: The *perception threshold* is the minimum concentration of on odoriferous compound detected by 50% of tasters in a triangular test, whereas the smell of that compound is not necessarily identified. The *recognition threshold* is the concentration at which the smell of a compound can be identified and the *preference threshold* is the maximum level at which a compound may be present without being perceived as negative (*4*). Thresholds of volatile compounds differ from very low pg/L and ng/L to mg/L levels.

Besides the sensory threshold of a compound the intensity of percieved aroma as a function of concentration is essential to evaluate its odor potential. Figure 1 gives an example of the dependency of the concentration on the perceived odor intensity for two compounds A and B.

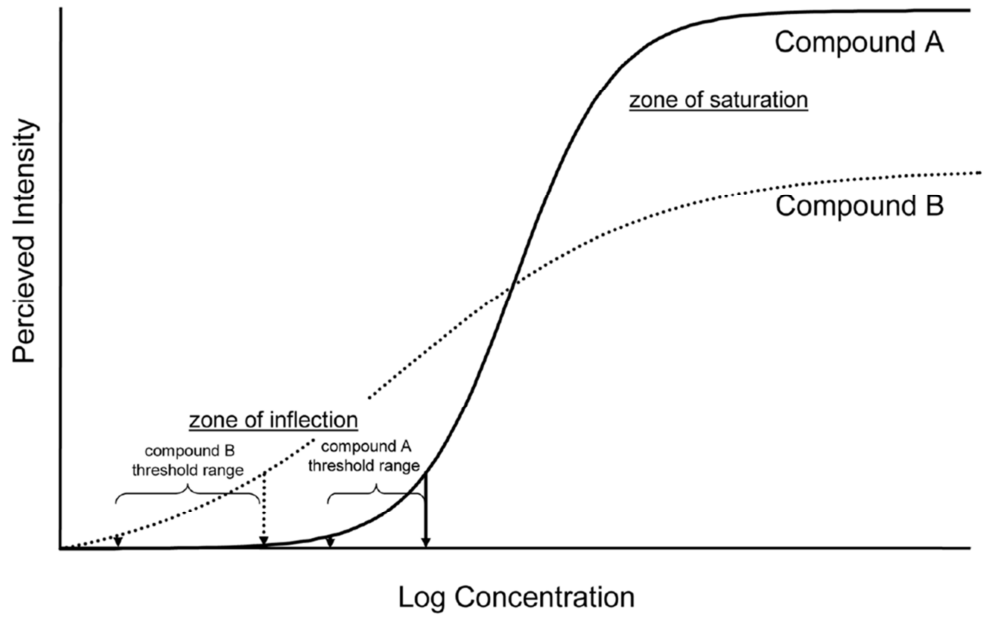

Figure 1: Psycometric functions of two compounds A and B: Correlation of the concentration and the perceived odor intensity (adapted from *(75)*).

The perceived impact of a compound is also dependent on its concentration. At a concentration close to the perception threshold a compound might not show any noticeable effect, whereas with increasing levels the effect becomes more defined. However, the sensory impression of a compound may also differ with concentration, as illustrated in Table 1 for *trans*-2-nonenal.

Table 1: Differing sensory impression of trans-2-nonenal as a function of concentration in aqueous solution (adapted and modified from (*3*)).

Odor descriptor	Concentration (µg/L)
Threshold	0.08
Slightly plastic-like	0.2
Woody	0.4 - 2.0
Fatty	3 - 16
Unpleasant oily	30 - 40
Strong cucumber	1000

In complex mixtures the odors of compounds may stay distinct, suppress each other or synergistically create another sensory impression. Even compounds present below their threshold levels can therefore affect the perceived aroma of wine.

The very complex wine matrix contains in excess of 700 volatile compounds. A large number of these substances contribute to the aroma of wine. Wine volatiles originate mainly from three sources: the grapes used, wine microbes (for example fermentation) and the maturation process (e.g. extraction of compounds from wood). Even though some aroma compounds originate directly from the grapes, the fermentation process with yeast plays a particularly important role in the formation of wine aroma. Yeast predominantly metabolizes sugar to alcohol and carbon dioxide. However, the formation of major or minor odor active metabolites from for example sugar and amino acids, and the modification of grape-derived compounds, such as the release of aroma compounds from odor inactive grape-derived glyco- and cysteine conjugated precursors during fermentation are essential for the development of wine aroma. After yeast, the second most important microorganisms in winemaking are lactic acid bacteria (LAB). LAB are primarily used to conduct malolactic fermentation (MLF) during or after alcoholic fermentation. The main goal of MLF is the reduction of acidity by the conversion of harsh tasting L-malic acid to milder-tasting L-lactic acid. In addition, MLF leads to alteration of wine aroma by the production of aroma active compounds or by alteration of compounds derived from grapes or alcoholic fermentation (*2*, 3, *5*). The most important groups of volatile compounds found in wine are discussed below.

3.2 Classes of wine volatiles

3.2.1 Alcohols

Ethanol is the most abundant volatile compound in wine. Its content in wine varies between 7 – 16% (v/v). The ethanol content of wine has an impact on the solubility and volatility of odor active compounds and therefore significantly affects the sensory perception of wine (*6*). As a result of its chemical properties, ethanol undergoes esterification with organic acids, leading to the production of ethyl esters, such as ethyl acetate, which contributes an unpleasant solvent-like odor if present in high concentrations. Furthermore, the reaction of ethanol with hydrogen sulfide may potentially lead to the formation of the potent compound ethanethiol, which is responsible for a sulfurous off-odor. Methanol is exclusively formed during enzymatic degradation of grape-derived pectin and therefore always occurs in wine. The concentration of this toxic compound in wine is, however, very low.

Alcohols with more than 2 carbons are called higher alcohols, also referred to as fusel alcohols. Yeast produces higher alcohols during fermentation either from sugar or from

grape-derived amino acids via the Ehrlich reaction. For example 2-methylpropanol, 3-methylbutanol and 2-methylbutanol are predominantly formed via this pathway. These alcohols enhance the complexity of a wine when present in low levels (< 300 mg/L), but at higher concentrations result in pungent odors suppressing the fruitiness and elegance of the wine (*3, 4*).

The C_6-alcohols such as hexanol and cis-3-hexenol are associated with green, herbaceous odors. These alcohols occur especially at higher levels in wines produced from unripe grapes and are formed by oxidation of the corresponding aldehydes, which in turn are thought to stem from the enzymatic cleavage of oxidized linoleic and linolenic acids (*3, 4*).

Another important alcohol is 1-octen-3-ol, which has an odor reminiscent of mushrooms and is especially effective in wines made of grapes infested with *Botrytis cinerea* (*4, 7*). This alcohol is also a well-known metabolite of many molds such as *Aspergillus* and *Penicillium* (*8*).

3.2.2 Aliphatic fatty acids

The most abundant organic acids in wine such as tartaric acid, malic acid and lactic acid are not volatile, although their concentrations may still have an effect on the aroma by playing a role in the release of aroma compounds from wine. A wide range of volatile and semi-volatile aliphatic acids are also present in wine. Acetic acid is of prime importance, as it contributes to around 90% of the volatile acidity (VA). All wines contain acetic acid, since small amounts of this compound are produced by yeast. Higher concentrations, however, originate from microbial spoilage, for instance by LAB or *acetobacter* species. Higher levels of propanoic acids and butanoic acids are also associated with microbial contamination. The longer chain hexanoic, octanoic and decanoic acids are metabolites of yeast activity. At low levels these acids contribute to the complexity of wine aroma. However, at higher concentrations they lead to objectionable rancid, pungent, cheese and fat-like aromas (*9*). Furthermore, these compounds can act as fermentation inhibitors if present at high mg/L (ppm) levels and are therefore believed to one possible cause of stuck fermentation (*2*, *4*).

3.2.3 Esters

The contribution of esters to wine aroma is very important. Wine esters are mostly formed enzymatically during fermentation, although chemical esterification also occurs. The amount of esters formed during fermentation depends on the esterase activity of the yeast, fermentation temperature and the degree of must clarification. Chemical esterification

involves the reaction of alcohols and acids and is an equilibrium reaction. Often levels of esters increase during wine aging. Especially esters of higher molecular weight acids tend to increase as a function of time, since they are present at low levels after fermentation (e.g. succinic acid esters). However, ethyl esters of low molecular weight acids are generally formed in excess during fermentation. As the esterification reaction is reversible, these esters hydrolyse during wine aging, leading to a decrease in their levels. Factors contributing this hydrolysis reaction include high temperature and low pH (*4, 10*).

3.2.4 Carbonyl compounds

Aldehydes are oxidation products of alcohols and play, with a few important exceptions, a minor role in wine aroma. Acetaldehyde (ethanal) is mainly formed by yeast during fermentation and contributes to an oxidized wine aroma. It reacts with sulfur dioxide and other wine constituents such as some phenolic compounds. Insufficient addition of sulfur dioxide during vinification results in elevated levels of free acetaldehyde, which causes “flatness” in wines. The oxidized aroma induced by free acetaldehyde is often recognized as an odor of freshly cut apples, which disappears after sulfur addition. This aldehyde also plays an important part in the typical aroma of brandy and sherry. The bouquet of some wines is also affected by higher aldehydes. This can be observed when the fruitiness of wine is reduced following sulfuring due to the reaction of sulfur dioxide with these aldehydes. The C_6 aldehydes such as hexanal and cis-3-hexenal confer herbaceous odors and originate from the grape and are precursors to the C_6 alcohols. Some aromatic aldehydes such as vanillin and cinnamic aldehyde originate from wood contact *(2,4)*.

The most important ketone in wine is the diketone diacetyl (2,3-butandione). Minor amounts of this compound are formed during alcoholic fermentation. However, higher levels originate from LAB activity during MLF or as a consequence of microbial spoilage. Diacetyl has odor descriptors of “sweet”, “buttery” and “butterscotch”, which are perceived as a pleasant aroma when present at low concentrations, but leads to an objectionable off-flavor at higher levels. Lactic acid bacteria metabolize citric acid to pyruvic acid, which is then reductively decarboxylised via diacetyl and acetoin to form 2,3-butanediol. Note that diacetyl has a much lower odor threshold than 2,3-butanediol and therefore affects wine aroma, whereas the odor threshold of 2,3-butanediol is rarely exceeded in wine (*3*, *4, 11*).

3.2.5 Lactones and furans

Lactones can have an important effect on wine aroma. These compounds are cyclic esters which are formed via intra molecular condensation of an alcohol and carboxylic acid.

Saturated γ-lactones are also called dihydro-furans. Some lactones can be formed from hydroxycarboxylic acids during fermentation. For instance γ-hydroxybutanoic acid, which is formed by deamination and decarboxylation of glutamic acid, rearranges to produce γ-butyrolactone (dihydro-3(H)-furan-2-one). Other lactones are linked to specific grape cultivars, such as 2-vinyl-dihydrofuran-2-one in Riesling and Muscat, or 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol) in Merlot and *Vitis lambruso* wines. The lactone sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) in particular is associated with botrytized and fortified wines. Sotolon can also be formed by condensation of α–keto butyric acid and ethanal. Other important compounds are the *cis* and *trans* isomers of 3-methyl-γ-octalactone, also known as “oak lactones” or “whiskey lactones”. These compounds are present at ppm levels and contribute strongly to the oaky aroma of wooded wines. Other compounds of this class can arise from saccharide degradation and through the Maillard reaction (*3, 4, 12*).

3.2.6 Terpenes

Terpenes consist of isoprene (C_5) units, and the most important classes in wine are monoterpenes (C_{10}, 2 isoprene units) and sesquiterpenes (C_{15}, 3 isoprene units). Furthermore, C_{13}-norisoprenoids also have important odor properties. The term terpenoid is used to refer to a terpene compound which has been chemically modified, for instance by oxidation or rearrangement. In the following discussion the term terpene will be used to include all terpenoids for the sake of simplicity (*4*). Of the approximately 40 monoterpenes identified in wine, the most important odoriferous compounds are linalool, α-terpineol, nerol, geraniol, citronellol and hotrienol. Linalool and citronellol are of special importance, since their olfactory thresholds are in the lower µg/L range. Terpenes essentially determine the aroma of Muscat grapes and wines such as Muscat d’Alexandria, Muscat d’Alsace and Muscat á Petits Grain. They are also involved in the “Muscat” characteristics of Alsatian and German grape cultivars such as Gewürztraminer, Riesling, Pinot gris, Auxerrois, Scheurebe and Müller-Thurgau. The aroma of Viognier and Muscadelle can also be affected by terpenes. However, in other famous grape cultivars such as Sauvignon blanc, Syrah, Cabernet Sauvignon, Merlot and Cabernet franc terpenes are usually present under their olfactory thresholds, and therefore play only a minor role in the aroma profiles of these wines. Other monoterpene derivatives containing alcohol (3,7-dimethyl-1,5-octadien-3,7-diol), aldehyde (geranial and linalal), acid (*trans*-geranic acid) and ester groups (geranyl and neryl acetate) are also present in wine (*3, 4*).

To a large extent terpenols (including diols and triols) such as linalool, nerol, geraniol, citronellol and α-terpineol are present in grapes as non-volatile, odourless glycosides. Four types of attached sugar moieties are known: the monosaccharide β-D-glucose and the

disaccharides α-L-arabinofuranose-β-D-glucopyranose, α-L-rhamnopyranose-β-D-glucopyranose, β-D-xylopyranose-β-D-glucopyranose and β-D-apiofuranose-β-D-glucopyranose. Other important wine volatiles such as hexanol, 2-phenyl ethanol, benzyl alcohol, C_{13}-norisoprenoids and volatile phenols (e.g. vanillin) are similarly present in grapes as glycosides (*13, 14*). As the glycosylated forms of these compounds are more water soluble than the free forms, they act as carriers for transport and accumulation of these compounds in plants. In non-Muscat grape varieties the ratio of glycosylated terpenols to the free form is 1:1, whereas in some Muscat cultivars the levels of the glycosylated form can be 5 times higher. During fermentation the aglycone can be enzymatically released. Glycosidase enzymes used in this conversion may originate from the grapes, yeast or bacteria. Chemical acid hydrolyses also occurs in wine, albeit plays only a minor role in the levels of the free aglycones in wine (*15*).

C_{13}-norisoprenoids originate from the oxidative degradation of carotenoids (C_{40} terpenes) and are classified into megastigmanes and non-megastigmanes. Some megastigmanes such as *β*-damascenone and *β*-ionone exhibit very low perception thresholds (ng/L) in wine. *β*-Damascenone has odor descriptors of “flowery”, “tropical fruit” and “stewed apples” and *β*-ionone is characterized by an odor reminiscent of “violets”. Both compounds are present in all grape varieties and can be formed from several precursor compounds. The most important non-megastigmane is 1,1,6-trimethyl-1,2-dihydronaphtalene (TDN). It has a distinctive “kerosene” odor and contributes significantly to the “petroleum” smell of old Riesling wines (*4, 16*).

3.2.7 Volatile phenols

Volatile phenols are most often related to the objectionable “phenolic” character. The main compounds associated with this defect are 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol (*17*). The odor of vinyl-4-phenol is described as reminiscent of “pharmaceuticals”, “gouche paint” and “Band Aid®”, whereas 4-ethylphenol induces an odor of “barnyard” and “sweaty saddle”. These compounds contribute more to unpleasant odors than the other two volatiles phenols mentioned, which have odor descriptors of carnations (4-vinylguaiacol) and “smokey”, “spicy” (4-ethylguaiacol) (*4*). These volatile phenols are primarily formed from the cinnamic acids ρ-coumaric and ferulic acid by the highly specific cinnamate decarboxylase enzyme of *Saccharomyces cerevisiae* during fermentation. The production of 4-vinylphenols by cinnamate decarboxylase is inhibited by other phenolic compounds (e.g. procyanidins), which results in much lower concentrations of 4-vinylphenols in red wines compared to white wines. The amount of 4-vinylphenols formed in white wine depends on the cinnamate decarboxylase activity of the yeast and on the concentration of

the precursors, coumaric and ferulic acid. The concentrations of these two cinnamic acids vary between different grape cultivars (*17-19*). Another very important source of volatile phenols is spoilage by the yeasts *Brettanomyces/Dekkera*. *Brettanomyces bruxellensis* is the predominant species found in wine. This yeast contains a cinnamate decarboxylase which is not inhibited by other phenolic compounds, and therefore converts large amounts of cinnamic acids to 4-vinylphenol and 4-vinylguaiacol. Moreover, a second enzyme produced by this yeast, a 4-vinylphenol reductase, catalyses the further reduction to ethyl-phenol and ethyl-guaiacol, respectively. This enzyme is completely absent in *Saccharomyces cerevisiae*. The sulfur dioxide content of wine in the barrel is crucial to avoid phenol taint of wine by *Brettanomyces*. A concentration of 30 mg/L sulfur dioxide is sufficient for the total elimination of this spoilage yeast (*4, 17, 19*). Additionally, these volatile phenols can originate from wood extraction during barrel aging. Oak extraction can for instance lead to high levels of (*iso*-)eugenol, which has odor descriptors of “spicy” and “clove-bud oil” (*3*).

3.2.8 Nitrogen containing compounds

In general, volatile nitrogen containing compounds play a less marked role in wine flavor. The exceptions are the 3-alkyl-2-methoxypyrazines which originate from amino acid metabolism in the vine and are very important aroma compounds in Cabernet Sauvignon, Sauvignon blanc and Cabernet franc wines. They have been also identified in many other grape cultivars, albeit typically under their recognition threshold. The three most important compounds in this group, 3-isopropyl-2-methoxypyrazine, 3-isobutyl- 2-methoxypyrazine and 3-*sec*-butyl-2-methoxypyrazine, have very low perception thresholds in the lower ng/L region and contribute to odors reminiscent of “green pepper”, “asparagus” and “earthy”. Levels of 2-methoxy-3-isobutylpyrazine are systematically higher than the other two methoxypyrazines. In Cabernet Sauvignon and Cabernet franc wines this compound may contribute to undesired herbaceous aromas, which occur in wines made from unripe grapes. 3-isobutyl-2-methoxypyrazine is located in the skin of grape berries, and therefore its concentration increases during mash fermentation. These herbaceous aromas associated with 3-isobutyl-2-methoxypyrazine play an essential role in determining the characteristic aroma of Sauvignon blanc wines (*4, 20, 21*).

Other volatile nitrogen compounds are primarily related to off-flavors, such as some amines and amides originating from bacterial spoilage (*3*) or 2-aminoacetophenone (2AAP), which is a key compound associated with the atypical aging off-flavor (*22*). High levels of 2AAP are linked to several factors such as reduced nitrogen fertilization, drought stress, hot conditions and early harvest (*23, 24*). 2AAP together with methylanthranilate contribute also to the

"foxy-taint" of some American hybrids (*25, 26*). Indole and skatole have been reported to cause plastic-like objectionable flavors (*27*).

It has been reported that thiazoles and oxazoles contribute to the aging aroma of wine (*28-30*). The mechanism of the formation of these compounds is not yet fully understood (*29-31*), although they might be formed in a Maillard-type reaction between carbonyl or dicarbonyl compounds and amino acids.

3.2.9 Sulfur containing compounds

Most volatile sulfur compounds present in wine are associated with objectionable odors, although some thiols contribute positively to the varietal aroma of certain grape cultivars. Volatile sulfur compounds in wine mainly originate directly or indirectly from yeast metabolism. Other possible sources of these compounds include formation from residues from wine sprays containing elemental sulfur and thermal or photochemical reactions. Volatile sulfur compounds can be grouped into low-boiling (<90°C) and high-boiling (>90°C) compounds (*4*). Low-boiling sufur compounds such as hydrogen sulfide, methanethiol and ethanethiol are predominantly responsible for reductive off-flavors. These compounds have odor descriptors of "rotten egg" and "sewage" amongst others. Of these compounds, hydrogen sulfide plays the most important role in wine aroma. It is formed in yeast by enzymatic action involving the reduction of sulfates and the biosynthesis of the sulfur containing amino acids such as cysteine and methionine. Extremely high levels of hydrogen sulfide are formed under conditions of nitrogen deprivation, as the yeast uses sulfur containing amino acids to satisfy its nitrogen demands. The addition of ammonium sulfate to must provides assimilable nitrogen to the yeast and therefore prevents the formation of hydrogen sulfide. In addition, methanethiol and ethanthiol can be formed from the corresponding alcohol and hydrogen sulfide (*2, 4, 32*). In contrast to most other sulfur compounds dimethyl sulfide (DMS) does not negatively influence wine aroma, but is considered to have a positive impact on the bouquet (*33*). This compound stems from the yeast metabolism of cystine, cysteine and glutathione. Amongst the high-boiling sulfur volatiles, which play only a minor role regarding reduction off-flavors, methionol is the most important. It is formed by deamination and decarboxylation of methionine according to the Erlich reaction (*4, 34*, *35*).

Certain thiols induce positive fruity aromas and contribute to the characteristic aroma of some grape varieties. Particularly the varietal aroma of Sauvignon blanc is (in addition to the contribution of methoxypyrazines) determined by 4-mercapto-4-methyl-pentan-2--one (4MMP), 4-mercapto-4-methyl-pentan-1-ol (4MMPOH), 3-mercapto-3-methyl-butan-1-ol (3MMB), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexanolacetate (A3MH) (*36*). These

thiols have the following odor descriptors: “boxtree” and “passion fruit” (4MMP); “passionfruit”, “grapefruit”, “gooseberry” and “guava” (A3MH & 3MH); and “cooked leeks” (3MMB) (*4, 36*). These compounds have also been reported to contribute to the aroma of other grape cultivars such as Riesling, Alsace Muscat and Chenin Blanc amongst others (*4*). All of these thiols are present in must as non-volatile *S*-cysteine conjugates. It is assumed that yeast originated β-lyase enzymes are responsible for the non-quantitative release of these thiols during fermentation (*37, 38*).

3.3 Malolactic fermentation and its impact on wine aroma

This discussion focuses exclusively on the effect of MLF on wine volatile composition and some overlap with the preceding section is thus unavoidable. Wine is a product of the fermentation of grape juice by yeast. Following this primary alcoholic fermentation, a secondary fermentation process, malolactic fermentation (MLF), can be conducted by lactic acid bacteria. However, MLF can also be performed simultaneously with primary fermentation by yeast. During these biological processes many chemical and biochemical reactions involving a wide variety of enzymes take place. In addition to these primary reactions (conversion of sugar to alcohol and malic acid to lactic acid) both forms of fermentation lead to other important chemical changes in grape must which affect wine properties such as flavor, mouth-feel, color and overall complexity. The importance of alcoholic fermentation is evident from comparison of the simplicity of grape must flavor compared to the complexity of wine flavor. Changes in wine aroma during alcoholic fermentation are mainly due to the production of volatile compounds by yeast and the modification of grape-derived compounds, especially the release of odor active compounds from non-volatile precursors (e.g. glyco- and cysteine conjugates). These interactions of yeast have been extensively studied during recent decades, since they determine the primary sensory properties of wine (*2, 32*). Intensive research has led to a broad understanding of the alcoholic fermentation process in wine, which has resulted in significant improvements in winemaking. However, research on the effect of the other important wine microorganisms, most noteably lactic acid bacteria, on wine flavor has long been neglected.

Due to its low pH, high ethanol concentration and low content of nutrients, wine offers rather unfavorable conditions for the growth of bacteria. The only LAB genera which are adapted to this harsh environment are *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*. *Oenococcus oeni* is the species most widely used for MLF. The main objective of malolactic fermentation is the conversion of malic acid into lactic acid, leading to the deacidification of wine. In addition to the reduction of acidity, MLF also results in a milder taste, enhanced mouth-feel and microbial stability (*39*, *40*). However, chemical and biochemical reactions

associated with MLF also have a significant impact on the volatile composition of wine, and therefore on its sensory properties. For instance, glucosidase, esterase and lipase enzymes originating from LAB were reported to contribute to the changes in wine aroma following MLF (*41-44*). Current knowledge on the changes in levels of odoriferous compounds related to MLF and will be briefly summarized below.

3.3.1 Carbonyl compounds

Diacetyl (2,3-butanedione) is associated with odor descriptors of “buttery” and “butterscotch” and is the most studied flavor compound associated with MLF. At low concentrations this compound can contribute to nutty and toasty aromas, while at high concentrations diacetyl leads to an intense objectionable “buttery” odor (5, *39*). Its perception is, however, highly dependent on the wine matrix, and when well balanced it imparts a significant stylistic odor contribution to malolactic fermented wines (*40, 45*). Diacetyl is formed via the metabolism of citric acid and can be further reduced to acetoin and 2,3-butanediol. These two reduction products have sensory thresholds of more than two orders of magnitude higher than diacetyl and therefore do not significantly affect wine aroma. The formation of diacetyl is determined by several factors which can be influenced by the winemaker. Firstly, the synthesis of diacetyl depends on the LAB strain used. In addition a lower inoculation rate, lower fermentation temperature, oxygen import into the wine, lower pH and a high citric acid concentration favor diacetyl production, whereas contact with active yeast (the lees) reduces the amount of diacetyl produced. Since diacetyl is a carbonyl compound it reacts similarly to acetaldehyde with sulfur dioxide in a reversible manner. Sulfuring can therefore reduce the buttery flavor of MLF wines, whereas a decrease of sulfur dioxide during storage results in the release of diacetyl and increases its effect on wine aroma (*40*, *45*).

It has been shown that glyoxal, methylglyoxal, hydroxypropanedial and 2,3-pentanedione are also produced by LAB (*46-48*) However, these compounds do not notably affect the wine aroma and can also be produced by yeast (except for 2,3-pentandione) (*32*). Furthermore, it has been shown that dicarbonyl compounds, especially diacetyl, can undergo Maillard-type reactions with amino acids to form heterocyclic aroma-active compounds such as thiazole derivatives, compounds known to be formed during wine aging (*29-31, 49*).

It has also been demonstrated that some wine LAB including *O. oeni,* are able to significantly reduce the levels of acetaldehyde in wine (*50-53*). This fact is particularly important from an oenological point of view as it allows reduction of sulfur dioxide levels in wine. Acetaldehyde together with hexanal, cis-hexen-3-al and trans-hexen-2-al impart green, grassy, vegetative aromas to wine. The degradation of aldehydes following MLF could therefore be responsible for the observed reduction of green/vegetative aromas (*54*). Furthermore, decreased levels

of 2-methyl-1-butanal and 3-methyl-1-butanal have been reported following MLF of Chancellor wines (*53*). In contrast the concentrations of 11 aldehydes in Pinotage and Syrah wines did not show significant differences following MLF (*55*).

3.3.2 Esters

Most studies on the impact of MLF on wine flavor have been focused on changes in the concentrations of esters. There are several conflicting reports of increasing (*53, 56-58*) or decreasing (*59*) of ester concentrations following MLF. However, the general consensus seems to be that ethyl esters tend to increase, whereas acetate ester concentrations decrease (*60, 61*). Several factors such as grape variety, bacterial strain, wine composition, vintage and geographical origin affect ester concentrations (*52, 61*).

Studies have indicated that wine associated lactic acid bacteria exhibit a wide arsenal of enzymatic activities (*10*). Several studies examined the esterase activity of commercial MLF strains (*41, 42*), while the esterase enzyme from *O. oeni* has also been characterized (*62*).

The formation of especially ethyl lactate is strongly linked to MLF, since the decarboxylation of malic acid results in high concentration of lactic acid in wine. This leads to higher concentrations of ethyl lactate due to chemical esterification. In addition, ethyl lactate was also found to be formed enzymatically by LAB (*11, 63*).

3.3.3 Higher alcohols

Although some wine volatile profiling studies have shown that several alcohols such as 1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol, 3-methyl-1-pentanol, 3-ethoxy-1-propanol and 2-phenylethanol increase following MLF, the process seems not to systematically influence the concentration of higher alcohols in wine (*52, 55, 57, 58, 64*). The production of higher alcohols in wine as a result of MLF is strain dependent (*55, 57*).

3.3.4 Volatile aliphatic fatty acids

Acetic acid is the most important volatile acid in wine and between 0.1 - 0.2 g/L is produced during MLF (*11*). Since the perception threshold of this compound is 0.7 g/L (*9*), this small increase is acceptable and MLF does not significantly affect the perception of acetic acid in wine.

Yeast lipase enzymes are responsible for the formation of longer chain aliphatic fatty acids. Although the lipolytic system in wine associated LAB is not well understood (*42*, *43*), it has been shown that the lipase activity of these bacteria is limited (*65*). However, an increase in volatile fatty acids due to MLF has been reported in several studies (*52*, *55, 57*), which could also be linked to the hydrolysis of the corresponding esters.

3.3.5 Glycosylated compounds

Analogously to yeast, wine LAB are capable of releasing glycosylated aroma compounds. The glycosidase activity of *O. oeni* strains has been reported to be substrate specific and is determined by wine conditions such as pH, ethanol content and temperature (*44*). Although numerous studies confirmed the release of aglycones due to glycosidase activity of LAB (*66-69*), it has also been reported that the concentrations of these compounds decreased following MLF (*69, 70*). Possible explanations for this phenomenon could be the formation of stable linkages of these compounds with bacterial polysaccharides (*70*), and the partial metabolization of the aglycones by LAB (*66*).

3.3.6 Volatile phenols

Laboratory studies have shown that some wine LAB strains posses the enzyme hydroxycinnamic acid decarboxylase, which produces the volatile phenols vinyl and ethyl phenol from p-coumaric and ferulic acids, respectively (*71*). However, only very small amounts of 4-vinylphenol and no 4-ethylphenol were actually formed from *p*-coumaric acid in white wine (*17*). Moreover, *O. oeni* is not able to decarboxylate *p*-coumaric acid, and therefore does not contribute to the formation of 4-vinylphenol (*71, 72*).

3.3.7 Sulfur containing compounds

The impact of MLF on sulfur containing compounds is not well understood. It has been shown that *O. oeni* is able to form hydrogen sulfide, methanethiol, dimethyl disulfide, methional and methionol in wine-like media following the addition of methionine and glutathione in concentrations far above their normal concentrations in wine (*73*). Increasing levels of methionol due to MLF have been reported in studies which focused on the profiling of major wine volatiles (*53, 56, 57*). Pripis-Nicolau and co-workers demonstrated the catabolism of methionine by *O. oeni* in laboratory media, resulting in increased levels of methanethiol, dimethyl disulfide, methionol and 3-(methylsulphanyl)propionic acid. However, in red wine only 3-(methylsulphanyl)propionic acid concentration increased significantly. This

compound is described by “chocolate” and “roasted” odors and could contribute to the enhanced aromatic complexity of MLF wines (*49*). Vallet and co-workers described the pathways that lead to the production of these compounds from methionine in *O. oeni* (*74*). They also reported an alcohol dehydrogenase in *O. oeni* which is involved in the conversion of methional to methionol (*35*). As mentioned previously, the formation of thiazoles and other heterocyclic compounds such as oxazoles from carbonyl and dicarbonyl compounds such as diacetyl have been demonstrated in wine. These compounds are considered to be linked to MLF, as the formation of the precursor diacetyl and other dicarbonyl compounds such as glyoxal, methylglyoxal, hydroxypropandial and 2,3-pentanedione is strongly associated with MLF (*29-31*).

This discussion points out the impact of malolactic fermentation on wine aroma. Although research has increasingly focused on this topic, a complete understanding of all the processes causing changes in the volatile composition and consequently flavor of wine is still lacking. In-depth investigation could therefore increase knowledge in this regard.

3.4 References

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4

Investigation of the volatile composition of Pinotage wines fermented with different malolactic starter cultures using comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS)*

*This chapter has been accepted for publication to Journal of Agricultural and Food Chemistry, (October 2011, dx.doi.org/10.1021/jf2028208) and is therefore written according to the style of this journal. List of Authors: Jochen Vestner, Sulette Malherbe, Maret du Toit, Hélène H. Nieuwoudt, Ahmed Mostafa, Tadeusz Górecki, Andreas G.J. Tredoux, André de Villiers

4.1 Introduction

Malolactic fermentation (MLF) is an important part of the vinification process of especially red wines. During MLF, lactic acid bacteria (LAB) facilitate the conversion of harsh-tasting malic acid to milder lactic acid. The resultant reduction in acidity and increase in pH improve the "mouth feel" of the wine (1). Furthermore, the decrease in levels of malic acid enhances the biological stability of the wine (2, 3).

Besides deacidification of wine, MLF also results in the production of volatile metabolites, as well as the modification of aroma compounds and flavour precursors originating from grapes and alcoholic fermentation, thereby influencing aroma of the final wine (4). As a result, MLF effectively offers winemakers an opportunity to modify the sensory properties of their product. It has been shown that LAB metabolism can have an impact on the final concentrations of different wine volatiles, including esters (5), alcohols (5), volatile phenols (6), terpenoids (7, 8), and sulphur compounds (9). The interaction of MLF bacteria with wine chemical constituents, however, is influenced, amongst others, by the wine type, the grape variety (10, 11), prevailing physico-chemical factors and the bacterial strain used to induce MLF (4, 6, 12-16). As a result of the low pH, high alcohol concentration and low nutrient levels associated with the wine matrix, only four LAB genera are known to be able to survive in wine. Three of these four, Lactobacillus, Leuconostoc and Pediococcus, are usually responsible for wine spoilage, while Oenococcus oeni is the preferred species for MLF (3, 17).

Previous research on the aroma modification of wine as a function of MLF was mainly focused on diacetyl (2,3 butanedione). This compound, in addition to acetic acid, acetoin, 2,3-pentanedione and 2,3-butanediol, is formed through citric acid metabolism by LAB and is one of the most important aroma compounds formed during MLF (10, 18). While diacetyl has a characteristic buttery aroma at higher concentrations, it can contribute to nutty and toasty aromas at lower concentrations (2, 5). The sensory impact and methods for diacetyl management in wine have been comprehensively studied (5, 19, 20) and reviewed by several authors (2, 10, 18, 21, 22).

In addition to an increase of buttery aroma, other alterations of aroma, such as the reduction of vegetative, green aromas or changes in perceived fruitiness, have been reported (4, 23). The reasons for these alterations of wine aroma are still not fully understood, since limited research has focused on the changes of volatile composition as a function of MLF. Levels of wine esters have been shown to vary following MLF, with some authors reporting increased (6, 14, 15, 24), while others lower concentrations for these compounds (12). Acetaldehyde, which can contribute together with hexanal, cis hexen 3 al and trans hexen 2 al to green,

grassy and vegetative aromas in wine, has been shown to be present at lower levels following MLF (25). The levels of several alcohols have also been shown to increase during MLF (13, 14, 16, 24). Monoterpenes, norisoprenoids, hydrocarbons and phenolic compounds can be released from their odourless glycoconjugated precursors by either acid or enzymatic hydrolysis. During alcoholic fermentation yeast provides glycosidases (26). Though similar enzyme activity for O. oeni has been demonstrated (2, 27, 28), a decrease of some of these compounds has been reported following MLF (6, 14). It is clear that MLF certainly does affect the aroma profile of wine, although a detailed description of this alteration in terms of chemical changes induced by MLF is still lacking.

Gas chromatography (GC) is the method of choice for the analysis of wine volatiles and has also been used for the investigation of the impact of MLF on wine volatile composition (6, 15, 29). Conventional GC methods do however display some limitations regarding selectivity and resolving power (peak capacity), especially when applied to the analysis of very complex mixtures such as wine. Comprehensive two dimensional gas chromatography (GC×GC) provides much higher resolution due to the combination of orthogonal separations using columns with different stationary phase properties (30, 31). The enhanced peak capacity, improved sensitivity, and structured retention patterns for compounds with similar chemical characteristics (30) make GC×GC a powerful tool for screening of the volatile composition of food products, as has been demonstrated for hazelnut and coffee (32, 33), fruits (34), olive oil (35), Cachaca (36) and wine (37-41). Schmarr et al. (39) used comprehensive two-dimensional gas chromatography-quadrupole mass spectrometry (GC×GC-qMS) to investigate the changes in volatile composition occurring due to micro-oxygenation of red wines. Robinson et al. (41) recently reported an untargeted method employing headspace solid phase microextraction in combination with GC×GC coupled to time-of-flight mass spectrometry (HS SPME GC×GC TOF MS) to investigate the influence of yeast strain, canopy management and field site on the volatile composition of Cabernet Sauvignon wines.

Previous research on the effect of MLF on volatiles in Pinotage wines (42, 43) utilized 1-dimensional GC with flame ionization (FID) and MS detection. This approach did demonstrate some limitations associated with uni-dimensional GC: primarily, the compounds identified and quantified were limited to those that can be separated on a single column and accurately quantified using these detectors. These compounds corresponded to major volatiles such as esters, alcohols and acids, as well as carbonyl compounds, which have previously been shown to undergo changes in concentrations as a result of MLF.

The relatively limited knowledge on the chemical changes induced in wine by MLF, which may be ascribed in part to the lack of relevant analytical data, clearly highlights the need for new methods of in-depth, comprehensive chemical profiling, as well as the importance of identifying impact odorants associated with MLF. This is especially true for Pinotage wines.

Pinotage is a uniquely South African grape variety cross bred from Pinot noir and Cinsaut (Hermitage) in 1925, and relatively little is currently known regarding the effect of MLF on Pinotage volatile composition.

In light of the above, the aim of this study was to apply GC×GC-TOF MS, and to exploit the benefits of this methodology for the in-depth qualitative and quantitative analysis of volatiles in Pinotage wines subjected to MLF. In order to study differences in volatile composition as a function of MLF conditions, wines produced under controlled conditions with different LAB starter cultures (42, 43) were analysed by GC×GC-TOF MS and data were analysed statistically to investigate the main effects

4.2 Materials and methods

4.2.1 Bacterial starter cultures

The three commercial starter cultures used in this study were Viniflora oenos® (O) and Viniflora CH16® (C), both from CHR Hansen (Hørsholm, Denmark), and Lalvin VP41® (V) from Lallemand (Stellenbosch, South Africa). All starter cultures were kindly donated by Lallemand and CHR Hansen.

4.2.2 Wine samples

Pinotage wine samples from the 2009 harvest were obtained from an earlier study (43), in which the impact of different MLF O. oeni starter cultures on wine aroma was assessed. Grapes were crushed, destemmed and 30 mg/L of sulfur dioxide was added. Alcoholic fermentation was conducted at 25℃ with the commercial yeast WE372 (Anchor Technologies, South Africa). Punch downs of the cap were done frequently. After pressing (at 2 °Brix), the wine was divided into different lots to produce triplicate biological repeats of the control wines (in which MLF was prevented through the addition of 0.25 g/L of lysozyme to the juice to inhibit LAB growth), and the wines produced using three different MLF starter cultures. Malolactic fermentations were performed in triplicate at 20℃, and were considered complete when the concentration of malic acid was below 0.3 g/L. The wines inoculated with starter cultures C and V completed MLF within 9 days, whereas those inoculated with starter culture O completed MLF within 12 days. All wines were racked from the lees, SO2 levels adjusted to 50 mg/L and stored at 0℃ for 2 weeks for cold stabilization before they were bottled as described before (43). All control and MLF wines were analyzed by GC×GC-TOF-MS after 8 months storage at 15℃.

4.2.3 Chemicals and materials

A series of C6 C18 n alkanes for the determination of linear retention indices were obtained from Sigma Aldrich (St. Louis, MO, USA). NaCl (ACS grade) was obtained from EMD Chemicals (Gibbstown, NJ, USA). Volatile standards (Table 1) were purchased from Sigma-Aldrich, Fluka (Zwijndrecht, Netherlands), Riedel-de Haën (Steinheim, Germany), and Merck (Darmstadt, Germany). For headspace solid phase microextraction (HS SPME), a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm fibre was used (Supelco, Belefonte, PA, USA).

4.2.4 Sample preparation

HS-SPME sampling was carried out as follows: 5 mL of the wine sample (pH adjusted to 3 using hydrochloric acid) was transferred to a 20 mL headspace crimp-top vial and spiked with 0.3 mg/L 2 pentanone as internal standard. 3 g sodium chloride (pre-heated to 250℃ and cooled to room temperature) was added to the vial together with a PTFE coated stir bar and the vial was capped immediately using a PTFE-lined septum and aluminium cap. The resulting saturated solutions were maintained while stirring at a temperature of 23℃ in a water bath before sampling. Each wine sample was submitted to HS-SPME sampling with stirring at 500 rpm for 5 and 30 minutes, respectively. Fibre blank and column blank analyses were carried out regularly to confirm that no sample carry-over occurred. Some hydrocarbons observed in the fibre blanks originated from the laboratory air. All chromatographic analyses were performed in duplicate.

4.2.5 Chromatographic conditions

An in-house developed GC×GC system consisting of an Agilent 6890 GC (Agilent Technologies, Palo Alto, CA, USA) equipped with a single jet, liquid nitrogen cryogenic modulator and coupled to a Pegasus III time-of-flight mass spectrometer (TOF MS) (LECO Corp., St. Joseph, MI, USA) was used for all analyses as previously described (44). Separation was carried out in the first dimension on a 30 m VF1-MS non-polar column (Varian, Mississauga, ON, Canada) with an internal diameter (i.d.) of 0.25 mm and a film thickness of 1.00 µm, which was coupled to a 1.5 m polar SolGel-Wax (SGE, Austin, TX, USA) second dimension column with an i.d. of 0.25 mm and a film thickness of 0.25 µm. A modulation period of 4 s was used with the cryogenic trap cooled to –196℃ using liquid nitrogen. The oven temperature program was as follows: initial temperature 40℃, kept for

0.2 min, ramped at 3°C/min to 170°C, then at 10°C/min to 250°C and held for 5 min. Thermal desorption and injection were performed using a split-splitless injector, operated at 260°C in the splitless mode, with a splitless time of 3 min. Helium was used as carrier gas at a constant flow of 1.5 mL/min. The transfer line between the GC and the MS was maintained at 250°C. Mass spectral acquisition was carried out in the mass range 35 450 amu at a rate of 100 spectra per second (ionization energy 70 eV). The ion source temperature was 225°C and the detector voltage was set to 1750 V. For initial data processing the automatic peak detection algorithm of the ChromaTOF software (LECO Corp. version 2.22) was used. Positive identification was performed by analysis of authentic standards. The remaining peaks were tentatively identified based on mass spectral comparison with the NIST 08 library. Using a series of n alkanes, first dimension retention indices (LRIcal) for each peak were automatically calculated by the ChromaTOF software. Experimental retention indices (LRIcal.) were compared to literature values (LRIlit) to confirm tentative peak identification based on mass spectra.

4.2.6 Statistical analysis

Analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test were carried out using STATISTICA v10 (StatSoft, Inc., Tulsa, OK, USA) to determine significant differences in sample means based on the 95% confidence level. For multivariate analysis the BiplotGUI package (45) of the open source software R (version 12.2.1) (46) was used. Peak area ratios of analytes relative to the internal standard were mean-centred and auto-scaled prior to construction of principal component analysis (PCA) biplots in R.

4.3 Results and discussion

4.3.1 HS-SPME-GC×GC-TOF-MS analysis of volatile composition

Wine contains a large number of diverse volatiles ranging widely in concentration, which makes analysis by one-dimensional GC, where sample components are typically separated by a single retention mechanism, challenging. In order to study both major volatiles and trace-level components in wine as a function of MLF, multiple analytical methods are often required (42, 43) to provide accurate quantitative data for a relatively limited number of compounds. In order to overcome these challenges, GC×GC was used in the current study. GC×GC combines two columns with different stationary phases and has been shown to be a particularly powerful separation method for the analysis of complex mixtures of volatiles, including wine (37-41, 47).

However, despite the enhanced selectivity and sensitivity of GC×GC, sample preparation remains a crucial part of the analytical procedure, especially if complex samples such as red wine are analysed. HS-SPME is commonly used for sample preparation prior to the analysis of wine volatiles and has been shown to be a simple, robust and sensitive method (38, 48-50). The DVB/CAR/PDMS SPME fibre used in this study has demonstrated its suitability for the extraction of a wide range of compounds (51-53). When profiling wine aroma, both minor and major compounds are of interest. Typically, extraction methods are optimized to provide either maximum sensitivity for trace level compounds (for example by removal of major volatiles which would otherwise obscure the analysis of minor constituents), or for analyses of major compounds (these methods do not provide the sensitivity required for low level analytes). When using SPME for screening both major and minor compounds, overloading sometimes occurs in one dimensional GC, but is even more prevalent in GC×GC (especially in the second dimension) because of refocusing of the bands in the cryogenic modulator. When excessive amounts of analytes are introduced into the GC×GC system, three phenomena combine to make accurate quantitation unreliable, if not impossible: the capacity of the modulator might be exceeded, which typically leads to significant injection band broadening and irregular injection band shapes; the second dimension column might be overloaded, which leads to distorted peaks; and finally, the linear dynamic range of the detector might be exceeded, which is particularly important when TOF-MS is used at high data acquisition rates. For these reasons, in the current work every sample was analysed using two different sets of HS SPME conditions. To extract the maximum amount of minor compounds, a 30 min extraction time was used. This time allowed the minor components to equilibrate with the fiber, thus maximizing the sensitivity. However, the major components overloaded the system under such conditions, which made their quantitation impossible. To overcome this problem, a 5 min extraction time was also used. The amounts of major components extracted under such conditions were significantly reduced, which eliminated overloading of the system and allowed accurate quantification of such compounds.

It should be noted, nevertheless, that the selective nature of HS-SPME does influence the compounds extracted from the wine matrix. This form of sample preparation is favourable for the more volatile wine constituents, but may not necessarily be suited to the analysis of higher-boiling compounds such as some terpenoids (37), for which alternative methods such as solid phase extraction (SPE) are better suited.

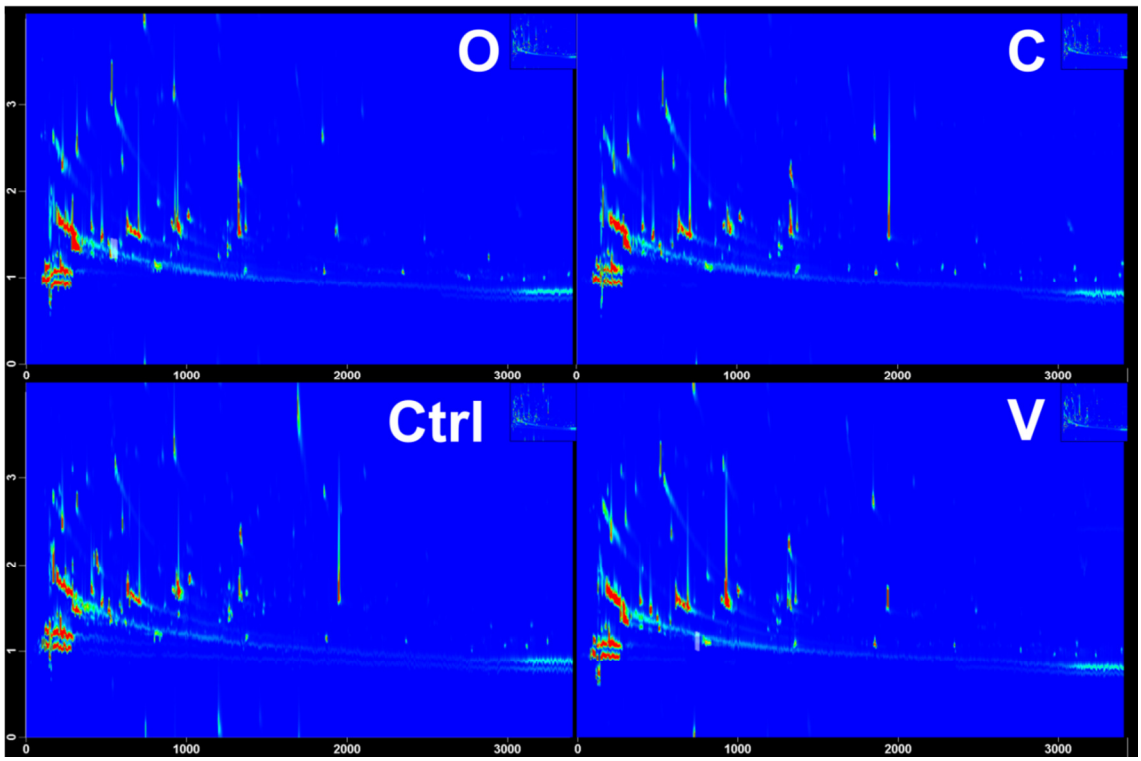

Figure 1: Analytical ion chromatogram (AIC) obtained for the control and the three MLF wines fermented with LAB starter cultures Viniflora oenos® (O), Viniflora CH16® (C) and Lalvin VP41® (V) using HS-SPME-GC×GC-TOF-MS (5 min extraction). The sums of unique ions (see Table 1) were used to generate the AIC.

Figure 1 presents contour plots obtained for the HS-SPME-GC×GC-TOF-MS analysis of the control and the three MLF Pinotage wines. Note that while some differences in the volatile profiles of the four wines are evident from this figure, the z-axis scale obscures further significant differences in the levels of minor constituents.

The orthogonal column configuration used in this study was a non-polar polydimethylsiloxane column in the first dimension providing separation mainly according to boiling point of the analytes, and a polar polyethylene glycol column in the second dimension providing separation based on differences in polarity. Therefore, more polar compounds were strongly retained in the second dimension, even leading to wraparound for compounds like ethyl S-lactate and to a larger extent the volatile acids. In general, these results are in agreement with previous reports utilising the 'normal' (i.e. apolar □ polar) column configuration for the GC×GC analysis of wine volatiles (40, 41, 38). Schmarr et al. (39) used a reversed, polar □ apolar, column combination for wine analysis, although significant breakthrough in the second dimension was reported under these conditions, resulting in multiple peaks being detected for numerous compounds.

The experimental set-up used here provided a significant improvement in the resolution of wine volatiles compared to conventional 1-dimensional GC. This is illustrated for a selected group of compounds in Figure 2. Linalool and 2 nonanol, as well as 2 methoxy 3 isopropylpyrazine (IPMP) and ethyl heptanoate, can be seen to co-elute in the first dimension because of their similar boiling points, but are separated in the second dimension due to differences in their polarity. The same is the case for nonanal and the unidentified compounds labelled unknown 2 and 3. On the other hand, nonanal and fenchone, as well as IPMP and unknown 1 are separated in the first dimension due to different boiling points, but co-elute in the second dimension because of their similar polarities. Clearly, co elution would inevitably occur in routine one dimensional GC screening methods utilizing a single stationary phase (typically polar) not optimised for separation of specific compounds.

Another benefit of GC×GC compared to one dimensional GC is the enhanced sensitivity, resulting from the re focusing of analytes in the modulator. This leads to narrower peaks and therefore larger signal to noise ratios in the second dimension (30). Excellent peak widths in the range of 100 ms for most analytes can be observed in Figure 2. Moreover, typical levels of IPMP in Pinotage are ~1 ng/L (54), which served to highlight the excellent sensitivity of the HS-SPME-GC×GC-TOF-MS method for selected trace-level compounds.

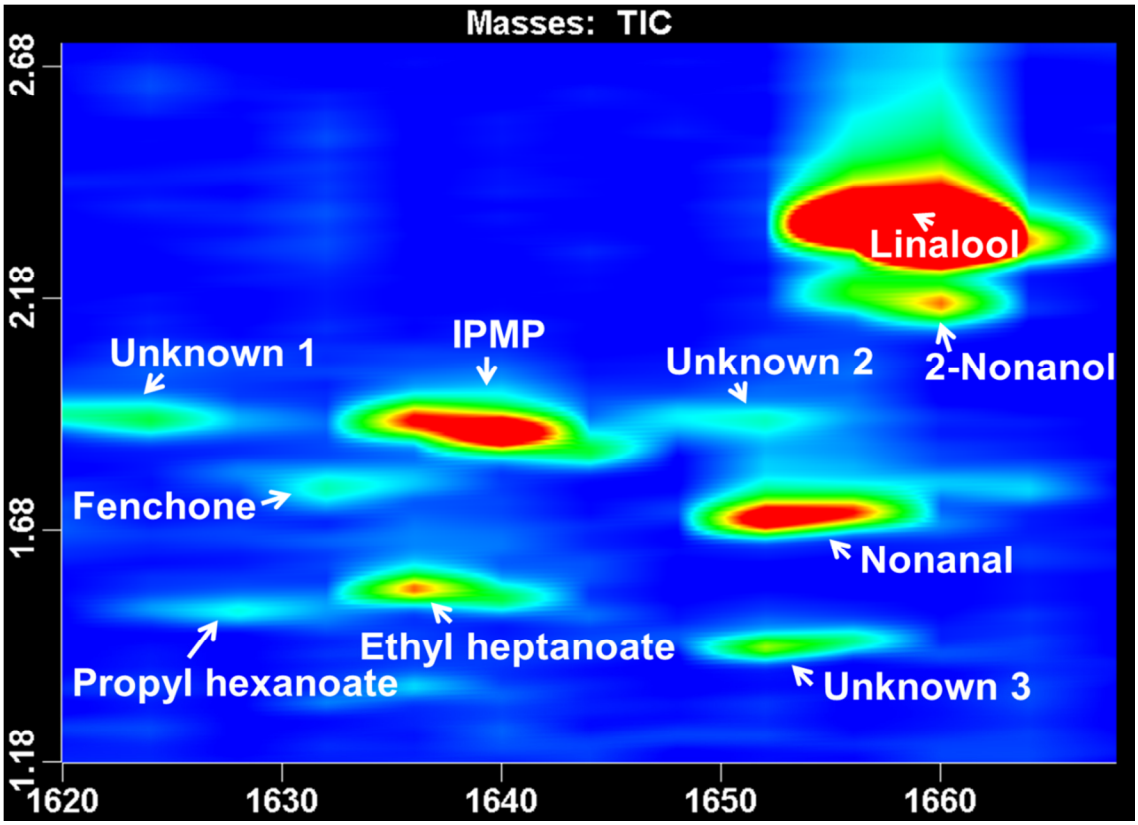

Figure 2: Total ion chromatogram of a wine fermented with starter culture O presenting the separation of selected volatiles by HS-SPME-GC×GC-TOF-MS (30 min extraction time).

Identification of the majority of peaks was based on comparison of deconvoluted mass spectra with the NIST 08 spectral library using ChromaTOF software, employing a minimum match factor of 70% as criterion. Furthermore, linear retention indices (LRI) were calculated using a homologous series of n-alkanes and compared with literature values. Taking into account that literature LRI values were determined by means of one dimensional gas chromatography, a relatively large maximum absolute difference of 30 between literature values and the experimental LRI values was used as criterion. In this manner, a total of 79 compounds were tentatively identified. In addition, authentic standards were used to positively confirm the identity of further 36 compounds (Table 1).

Since the goal of this work was to investigate differences in the levels of individual volatile compounds between wines as a function of MLF, special care was taken with the identification of compounds based on the abovementioned criteria. Compound identification was therefore confirmed manually in each instance. Although this conservative approach is necessarily time-consuming and resulted in a reduction in the number of compounds identified using an automated ChromaTOF search, we found this step essential to minimize the risk of possible incorrect identification and to improve statistical analysis and data

interpretation. This explains the relatively low number of tentatively identified compounds reported in this study compared to previous reports utilising GC×GC that were focused on screening of wine volatiles (38, 40). Table 1 provides a summary of all compounds identified using this strategy in the wine samples. Compounds identified included esters, alcohols, ketones, aldehydes, acids, acetals, furans, nitrogen containing compounds, and compounds with terpenoid character. They represent mainly grape- and fermentation derived wine volatiles, which are typically extracted using HS SPME methods (55).

Table 1. List of compounds identified and quantified in Pinotage wine samples by HS-SPME-GC×GC-TOF-MS. Alphabetic letters row wise indicate significant differences ($p<0.05$) in the sample means for triplicate biological repeats.

No.	Compound		1D RT	2D RT	MS match[1]	$LRI_{cal.}$[2]	$LRI_{lit.}$[3]	Unique mass	Ctrl[7,8] Average ±SD	V[7,8] Average ±SD	O[7,8] Average ±SD	C[7,8] Average ±SD
	Esters											
1	Formic acid, ethyl ester (Ethyl formate)	[4,5]	184	1.34	969	508	530	45	0.1548 ±0.010 [a]	0.1649 ±0.021 [a]	0.2117 ±0.032 [b]	0.2289 ±0.045 [b]
2	Acetic acid, methyl ester (Methyl acetate)	[5]	192	1.38	952	516	506	43	0.1629 ±0.011 [a]	0.2537 ±0.054 [b]	0.3129 ±0.022 [bc]	0.3339 ±0.047 [c]
3	Ethyl acetate	[4]	280	1.44	980	600	596	43	not quantified			
4	Propanoic acid, ethyl ester (Ethyl propanoate)	[5]	464	1.52	953	700	680	57	2.4040 ±0.109 [a]	3.7544 ±0.553 [b]	3.7631 ±0.338 [b]	4.3243 ±0.331 [c]
5	Acetic acid, propyl ester (Propyl acetate)	[5]	468	1.58	952	702	698	43	0.4509 ±0.028 [a]	1.5736 ±0.356 [b]	1.7971 ±0.170 [bc]	1.9623 ±0.237 [c]
6	Propanoic acid, 2-methyl-, ethyl ester (Ethyl isobutyrate)	[4,5]	588	1.44	920	746	743	71	0.4740 ±0.044 [a]	0.7433 ±0.094 [b]	0.7402 ±0.087 [b]	0.7974 ±0.102 [b]
7	Acetic acid, 2-methylpropyl ester (Isobutyl acetate)		624	1.54	939	760	767	56	not quantified			
8	1-Butanol, 3-methyl-, formate (Isoamyl formate)	[6]	672	1.73	789	778	777	55	0.0401 ±0.005 [n.s.]	0.0375 ±0.005 [n.s.]	0.0429 ±0.007 [n.s.]	0.0434 ±0.005 [n.s.]
9	Propanoic acid, 2-hydroxy-, ethyl ester (Ethyl lactate)		676	0.68	716	779	787	45	not quantified			
10	Butanoic acid, ethyl ester (Ethyl butyrate)	[4,5]	704	1.54	867	790	778	91	5.2829 ±0.254 [a]	15.2660 ±4.514 [b]	15.4990 ±3.532 [b]	15.5027 ±2.708 [b]
11	Propanoic acid, 2-hydroxy-, ethyl ester, (S) (Ethyl-S-lactate)	[4,5]	736	0.12	984	801	800	45	0.0869 ±0.006 [a]	0.4533 ±0.061 [b]	0.4814 ±0.027 [b]	0.5326 ±0.047 [c]
12	Acetic acid, butyl ester (Butyl acetate)	[5]	736	1.61	955	801	805	43	0.0769 ±0.010 [a]	0.2384 ±0.046 [b]	0.2785 ±0.030 [bc]	0.2891 ±0.054 [c]
13	2-Butenoic acid, ethyl ester (Ethyl 2-butenoate)	[5]	820	1.95	939	828	819	69	0.2864 ±0.011 [a]	1.1222 ±0.258 [b]	1.3006 ±0.102 [b]	1.3367 ±0.135 [b]
14	Butanoic acid, 2-methyl-, ethyl ester (Ethyl 2-methylbutanoate)	[4,5]	856	1.49	950	840	829	102	0.1007 ±0.007 [a]	0.1882 ±0.013 [b]	0.2666 ±0.003 [c]	0.2042 ±0.008 [b]
15	Butanoic acid, 3-methyl-, ethyl ester (Ethyl isovalerate)	[5]	860	1.5	920	841	824	88	0.1975 ±0.006 [a]	0.3005 ±0.016 [b]	0.2999 ±0.018 [b]	0.3484 ±0.006 [c]
16	1-Butanol, 3-methyl-, acetate (Isoamyl acetate)		928	1.62	925	863	856	43	not quantified			
17	1-Butanol, 2-methyl-, acetate (2-Methylbutyl acetate)		936	1.61	935	866	868	43	not quantified			
18	Pentanoic acid, ethyl ester (Ethyl pentanoate)	[4,6]	1004	1.57	913	887	881	88	0.0339 ±0.003 [a]	0.0553 ±0.012 [b]	0.0588 ±0.010 [b]	0.0659 ±0.008 [b]
19	Hexanoic acid, methyl ester (Methyl hexanoate)	[5]	1084	1.66	922	913	903	74	0.1522 ±0.008 [a]	0.1041 ±0.009 [c]	0.1003 ±0.008 [c]	0.1171 ±0.009 [b]
20	Butanoic acid, 3-hydroxy-, ethyl ester (Ethyl 3-hydroxybutyrate)		1108	0.6	939	920	949	43	not quantified			

No.	Compound		1D RT	2D RT	MS match[1]	$LRI_{cal.}$[2]	$LRI_{lit.}$[3]	Unique mass	Ctrl[7,8] Average ±SD	V[7,8] Average ±SD	O[7,8] Average ±SD	C[7,8] Average ±SD
21	1-Butanol, 3-methyl-, propanoate (Isoamyl propanoate)	[6]	1228	1.52	874	958	948	57	0.0240 ±0.003 [a]	0.0382 ±0.008 [ab]	0.0489 ±0.011 [b]	0.0524 ±0.005 [b]
22	Hex-5-enoic acid, ethyl ester	[4,6]	1276	1.76	930	973	965	60	0.0320 ±0.002 [a]	0.0558 ±0.011 [c]	0.0672 ±0.010 [bc]	0.0725 ±0.004 [b]
23	Butanoic acid, butyl ester (Butyl butyrate)		1312	1.53	942	984	978	71	not quantified			
24	Hexanoic acid, ethyl ester (Ethyl hexanoate)	[4]	1320	1.57	947	986	976	88	not quantified			
25	3-Hexen-1-ol, acetate, (E)-		1332	1.81	719	990	983	82	not quantified			
26	3-Hexenoic acid, ethyl ester (Ethyl-3-hexenoate)		1336	1.78	819	991	986	68	not quantified			
27	3-Hexen-1-ol, acetate, (Z)-		1344	1.83	898	994	987	67	not quantified			
28	Acetic acid, hexyl ester (Hexyl acetate)	[4,5]	1364	1.62	957	1000	990	43	0.5141 ±0.023 [a]	1.1137 ±0.242 [b]	1.1633 ±0.095 [b]	1.2256 ±0.225 [b]
29	Heptanoic acid, methyl ester (Methyl heptanoate)		1404	1.64	937	1013	1005	74	not quantified			
30	Ethyl 2-hexenoate	[5]	1452	1.79	957	1028	1023	97	0.0742 ±0.008 [a]	0.1211 ±0.013 [b]	0.1297 ±0.007 [b]	0.1454 ±0.030 [c]
31	Hexanoic acid, 2-ethyl-, methyl ester (Ethyl 2-ethylhexanoate)		1472	1.49	922	1035	1024	87	not quantified			
32	Propanedioic acid, diethyl ester (Diethyl malonate)		1504	3.11	899	1046	1038	115	not quantified			
33	Butanoic acid, 3-methylbutyl ester (Isoamyl butyrate)		1508	1.45	942	1046	1041	71	not quantified			
34	Pentanoic acid, 2-hydroxy-4-methyl-, ethyl ester	[6]	1512	2.77	848	1048	1043	69	0.0861 ±0.012 [a]	0.1013 ±0.011 [ab]	0.1131 ±0.014 [b]	0.1197 ±0.020 [b]
35	Propanoic acid, 2-hydroxy-, 3-methylbutyl ester (Isoamyl lactate)	[6]	1548	2.87	837	1060	1047	45	0.0250 ±0.005 [a]	0.0897 ±0.022 [bc]	0.0695 ±0.010 [c]	0.0918 ±0.022 [b]
36	Butanedioic acid, ethyl methyl ester		1624	3.21	915	1084	1070	115	not quantified			
37	Hexanoic acid, propyl ester (Propyl hexanoate)	[5]	1628	1.5	934	1085	1079	61	0.0215 ±0.002 [a]	0.0309 ±0.002 [b]	0.0320 ±0.003 [bc]	0.0327 ±0.004 [c]
38	Heptanoic acid, ethyl ester (Etyhl heptanoate)	[5]	1636	1.55	935	1087	1083	88	0.0753 ±0.004 [n.s.]	0.0763 ±0.006 [n.s.]	0.0809 ±0.005 [n.s.]	0.0830 ±0.009 [n.s.]
39	Octanoic acid, methyl ester (Methyl octanoate)	[5]	1720	1.62	942	1115	1108	74	0.0811 ±0.010 [a]	0.0638 ±0.003 [b]	0.0608 ±0.005 [b]	0.0555 ±0.023 [b]
40	Hexanoic acid, 2-methylpropyl ester (Isobutyl hexanoate)		1804	1.41	840	1143	1152	99	not quantified			
41	Butanedioic acid, diethyl ester (Diethyl succinate)	[4,5]	1844	2.77	968	1157	1151	101	2.0866 ±0.177 [a]	3.0600 ±0.228 [b]	3.0860 ±0.195 [b]	3.2183 ±0.051 [b]
42	Octanoic acid, ethyl ester (Ethyl octanoate)	[4]	1936	1.55	926	1187	1175	88	not quantified			
43	Methyl salicylate	[6]	1944	3.2	776	1190	1176	120	0.0253 ±0.003 [ac]	0.0141 ±0.003 [b]	0.0227 ±0.004 [c]	0.0267 ±0.004 [ab]

No.	Compound		1D RT	2D RT	MS match[1]	$LRI_{cal.}$[2]	$LRI_{lit.}$[3]	Unique mass	Ctrl[7,8] Average ±SD	V[7,8] Average ±SD	O[7,8] Average ±SD	C[7,8] Average ±SD
44	Benzeneacetic acid, ethyl ester (Ethyl phenylacetate)	[4,6]	2060	2.85	951	1231	1211	91	0.0647 ±0.007 [a]	0.0328 ±0.003 [b]	0.0364 ±0.007 [b]	0.0397 ±0.007 [b]
45	Acetic acid, 2-phenylethyl ester (2-Phenylethyl acetate)	[4,5]	2092	3.01	932	1243	1224	104	0.4673 ±0.137 [n.s.]	0.4264 ±0.061 [n.s.]	0.4316 ±0.088 [n.s.]	0.4353 ±0.106 [n.s.]
46	Hexanoic acid, 3-methylbutyl ester (Isopentyl hexanoate)		2092	1.46	966	1242	1253	70	not quantified			
47	Hexanoic acid, 2-methylbutyl ester (2-Methylbutyl hexanoate)		2104	1.44	922	1247	1236	99	not quantified			
48	Nonanoic acid, ethyl ester (Ethyl nonanoate)	[4,6]	2220	1.48	912	1289	1288	88	0.0161 ±0.003 [a]	0.0253 ±0.007 [b]	0.0269 ±0.006 [b]	0.0322 ±0.006 [b]
49	Ethyl 9-decenoate	[6]	2460	1.61	891	1379	1357	55	0.0262 ±0.008 [a]	0.0462 ±0.012 [b]	0.0478 ±0.009 [b]	0.0605 ±0.014 [b]
50	Decanoic acid, ethyl ester (Ethyl decanoate)	[4,5]	2488	1.47	918	1390	1367	88	0.1109 ±0.010 [a]	0.2975 ±0.026 [b]	0.3276 ±0.027 [b]	0.3116 ±0.034 [b]
	Alcohols											
51	1-Propanol	[5]	220	2.31	962	544	524	59	3.3180 ±0.267 [a]	4.7332 ±0.592 [b]	5.5978 ±0.352 [c]	5.2694 ±0.657 [c]
52	1-Propanol, 2-methyl- (isobutanol)	[4,5]	312	2.65	825	618	625	74	0.9690 ±0.135 [a]	1.5934 ±0.146 [b]	1.8160 ±0.141 [bc]	1.9612 ±0.125 [c]
53	1-Butanol	[4,5]	376	2.93	955	653	660	56	0.6411 ±0.066 [a]	0.8428 ±0.066 [b]	0.9640 ±0.060 [c]	0.9102 ±0.090 [c]
54	3-Buten-1-ol, 3-methyl-		520	3.78	923	722	728	39	not quantified			
55	1-Butanol, 3-methyl- (Isoamyl alcohol)	[4]	524	3.17	965	723	718	55	not quantified			
56	2-Pentanol, 4-methyl-	[5]	592	2.43	906	748	760	45	0.6999 ±0.076 [n.s.]	0.6518 ±0.048 [n.s.]	0.6128 ±0.051 [n.s.]	0.6899 ±0.033 [n.s.]
57	1-Pentanol (Amyl alcohol)	[5]	616	3.23	935	758	744	42	0.0711 ±0.018 [a]	0.1189 ±0.017 [b]	0.1248 ±0.006 [bc]	0.1360 ±0.028 [c]
58	2,3-Butanediol		644	2.48	945	768	748	45	not quantified			
59	1-Pentanol, 4-methyl- (isohexanol)	[5]	820	3.11	947	829	851	56	0.1327 ±0.011 [n.s.]	0.1399 ±0.016 [n.s.]	0.1555 ±0.007 [n.s.]	0.1564 ±0.017 [n.s.]
60	1-Pentanol, 3-methyl- (3-methylpentanol)	[5]	844	3.18	929	837	854	56	0.3140 ±0.027 [n.s.]	0.3014 ±0.022 [n.s.]	0.3287 ±0.030 [n.s.]	0.3270 ±0.029 [n.s.]
61	1-Hexanol	[4,5]	916	3.17	940	860	852	56	5.1824 ±0.042 [a]	4.5378 ±0.161 [b]	4.2983 ±0.027 [c]	4.3190 ±0.029 [c]
62	2-Heptanol	[6]	1016	2.45	894	891	877	45	0.0201 ±0.003 [n.s.]	0.0182 ±0.001 [n.s.]	0.0184 ±0.002 [n.s.]	0.0212 ±0.002 [n.s.]
63	1-Heptanol	[5]	1240	2.96	973	962	952	56	0.1981 ±0.027 [a]	0.1080 ±0.017 [b]	0.0973 ±0.012 [b]	0.0973 ±0.018 [b]
64	1-Octen-3-ol	[4,5]	1272	2.76	882	972	959	57	0.0951 ±0.011 [a]	0.0561 ±0.004 [b]	0.0625 ±0.009 [bc]	0.0638 ±0.010 [c]
65	2-Octanol, (R)-	[4]	1340	2.35	942	993	985	45	not quantified			
66	1-Octanol	[5]	1564	2.71	931	1065	1054	55	0.1082 ±0.009 [n.s.]	0.0901 ±0.021 [n.s.]	0.0909 ±0.013 [n.s.]	0.0803 ±0.027 [n.s.]
67	2-Nonanol		1660	2.17	948	1095	1084	45	not quantified			
68	Phenylethyl Alcohol	[4]	1684	3.71	954	1104	1082	91	not quantified			

No.	Compound		1D RT	2D RT	MS match[1]	$LRI_{cal.}$[2]	$LRI_{lit.}$[3]	Unique mass	Ctrl[7,8] Average ±SD	V[7,8] Average ±SD	O[7,8] Average ±SD	C[7,8] Average ±SD
	Ketones											
69	2,3-Butanedione (Diacetyl)		244	1.88	988	566	558	43	0.1766 ±0.003 [a]	0.2064 ±0.006 [b]	0.3755 ±0.013 [c]	0.2511 ±0.006 [d]
70	2-Butanone	[6]	256	1.47	949	577	582	72	0.0267 ±0.004 [a]	0.0121 ±0.001 [b]	0.0145 ±0.005 [b]	0.0161 ±0.005 [b]
71	2,3-Pentanedione	[4,6]	416	2.06	970	674	660	57	0.3459 ±0.022 [a]	0.1058 ±0.017 [b]	0.0972 ±0.007 [b]	0.1114 ±0.007 [b]
72	3-Penten-2-one	[5]	520	2.31	937	721	697	69	0.2566 ±0.026 [a]	0.1226 ±0.037 [b]	0.2934 ±0.032 [a]	0.3175 ±0.016 [a]
73	Methyl Isobutyl Ketone		528	1.59	882	724	730	58	not quantified			
74	2,3-Pentanedione, 4-methyl-		640	2.05	945	766	763	71	not quantified			
75	2-Heptanone	[6]	964	1.81	950	875	871	58	0.0405 ±0.002 [a]	0.0184 ±0.002 [b]	0.0103 ±0.002 [c]	0.0159 ±0.003 [bc]
76	1-Octen-3-one		1248	1.92	911	964	956	55	not quantified			
77	3-Octanone	[5]	1280	1.67	953	974	963	57	0.3742 ±0.052 [n.s.]	0.3030 ±0.024 [n.s.]	0.3279 ±0.136 [n.s.]	0.2973 ±0.039 [n.s.]
78	Acetophenone	[4]	1528	3.87	901	1054	1049	77	not quantified			
79	2-Pentanone		404	1.65	935	668	651	43	internal standard			
	Aldehydes											
80	2-Propenal (Acrolein)		160	1.33	976	485	470	56	not quantified			
81	Propanal, 2-methyl- (isobutanal)	[6]	216	1.26	929	539	532	41	0.0504 ±0.002 [ab]	0.0379 ±0.009 [a]	0.0662 ±0.016 [b]	0.0617 ±0.016 [b]
82	Butanal	[6]	252	1.4	854	573	575	72	0.0138 ±0.001 [n.s.]	0.0134 ±0.003 [n.s.]	0.0130 ±0.002 [n.s.]	0.0106 ±0.001 [n.s.]
83	Butanal, 3-methyl- (isopentanal)	[5]	344	1.49	938	635	628	58	0.4486 ±0.089 [n.s.]	0.4286 ±0.026 [n.s.]	0.5130 ±0.051 [n.s.]	0.5260 ±0.024 [n.s.]
84	Butanal, 2-methyl- (2-methylbutanal)		364	1.44	905	646	632	58	not quantified			
85	Hexanal	[4,6]	684	1.73	916	782	769	56	0.0348 ±0.002 [a]	0.0209 ±0.001 [b]	0.0199 ±0.003 [b]	0.0179 ±0.002 [c]
86	Benzaldehyde	[4]	1188	0.1	914	945	927	77	not quantified			
87	Octanal		1332	1.8	804	990	982	82	not quantified			
88	Benzeneacetaldehyde (Phenylacetaldehyde)	[5]	1444	0.3	954	1025	1012	91	0.2474 ±0.010 [a]	0.1948 ±0.013 [b]	0.1819 ±0.021 [b]	0.1854 ±0.029 [b]
89	Nonanal	[4,6]	1652	1.71	938	1093	1081	57	0.0853 ±0.017 [n.s.]	0.0505 ±0.009 [n.s.]	0.0616 ±0.012 [n.s.]	0.0760 ±0.019 [n.s.]
90	Decanal	[4,6]	1964	1.68	926	1196	1183	57	0.0217 ±0.005 [n.s.]	0.0192 ±0.005 [n.s.]	0.0227 ±0.004 [n.s.]	0.0264 ±0.007 [n.s.]

No.	Compound		1D RT	2D RT	MS match[1]	$LRI_{cal.}$[2]	$LRI_{lit.}$[3]	Unique mass	Ctrl[7,8] Average ±SD	V[7,8] Average ±SD	O[7,8] Average ±SD	C[7,8] Average ±SD
	Acids											
91	Butanoic acid		696	1.17	724	787	780	60	not quantified			
92	Hexanoic acid		1300	2.09	944	980	973	45	not quantified			
93	Octanoic Acid		1884	1.52	926	1170	1154	60	not quantified			
	Acetals											
94	1,3-Dioxolane, 2,4,5-trimethyl-		508	1.42	717	944	711	43	not quantified			
	Furans											
95	Furan, 2,5-dimethyl-		468	1.44	880	702	696	96	not quantified			
96	2-Furancarboxaldehyde (Furfural)		772	2.76	940	813	794	95	not quantified			
	Nitrogen containing compounds											
97	Pyrazine, 2-methoxy-3-(1-methylethyl)- [2-Methoxy-3-isopropylpyrazine (IPMP)]	[4]	1640	1.84	739	1089	1080	137	not quantified			
98	Pyrazine, 2-methoxy-3-(1-methylpropyl)- [2-Methoxy-3-sec-butylpyrazine (SBMP)]	[4]	1880	1.77	803	1168	1151	138	not quantified			
	Compounds with terpenoid character and others											
99	Benzene, (1-methylethyl)- [Cumene]		1112	1.59	915	921	907	105	not quantified			
100	Benzene, propyl-) [Isocumene]		1208	1.63	945	951	934	91	not quantified			
101	Camphene		1224	1.27	950	956	958	91	not quantified			
102	4-Heptanone, 2,6-dimethyl-		1240	1.48	878	961	951	56	not quantified			
103	5-Hepten-2-one, 6-methyl-	[6]	1272	2.04	901	972	938	43	0.0334 ±0.003 [a]	0.0029 ±0.000 [b]	0.0038 ±0.001 [b]	0.0036 ±0.000 [b]
104	5-Hepten-2-ol, 6-methyl-	[6]	1312	2.73	889	984	976	95	0.0246 ±0.004 [ab]	0.0229 ±0.003 [b]	0.0142 ±0.002 [c]	0.0275 ±0.004 [ab]
105	1,3-Cyclohexadiene, 2-methyl-5-(1-methylethyl)- [α-Phellandrene]		1388	1.4	863	1008	1007	93	not quantified			
106	1,6-Octadiene, 7-methyl-3-methylene- [ß-Myrcene]		1332	1.41	878	990	979	93	not quantified			
107	Cyclohexene, 1-methyl-4-(1-methylethenyl)- [α-Limonene]	[4,6]	1468	1.36	922	1033	1019	93	0.4025 ±0.061 [a]	0.1729 ±0.042 [b]	0.1374 ±0.036 [b]	0.1749 ±0.034 [b]
108	Benzene, butyl-		1540	1.61	896	1057	1036	91	not quantified			

No.	Compound		1D RT	2D RT	MS match[1]	$LRI_{cal.}$[2]	$LRI_{lit.}$[3]	Unique mass	Ctrl[7,8] Average ±SD	V[7,8] Average ±SD	O[7,8] Average ±SD	C[7,8] Average ±SD
109	Bicyclo[2.2.1]heptan-2-one, 1,3,3-trimethyl- [Fenchone]	[4]	1632	1.79	888	1086	1097	81	not quantified			
110	1,6-Octadien-3-ol, 3,7-dimethyl- [β-Linalool]	[4,5]	1660	2.31	834	1095	1081	71	0.1197 ±0.021 [a]	0.0840 ±0.008 [b]	0.0686 ±0.013 [b]	0.0669 ±0.011 [b]
111	4-Methyl-2-(2-methyl-1-propenyl)tetrahydro-2H-pyran [trans-Rose oxide]		1700	1.53	867	1108	1107	139	not quantified			
112	Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl- [Fenchol]		1728	2.43	855	1118	1099	81	not quantified			
113	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)- [L-Camphor]		1804	1.99	919	1143	1148	95	not quantified			
114	3-Cyclohexene-1-methanol, à,à4-trimethyl- [α-Terpineol]	[4]	1948	2.58	927	1191	1172	93	not quantified			
115	6-Octen-1-ol, 3,7-dimethyl-, [β-Citronellol]	[4,6]	2040	2.67	880	1224	1208	41	0.0968 ±0.015 [a]	0.0656 ±0.011 [b]	0.0622 ±0.016 [b]	0.0757 ±0.007 [ab]

[1] Mass spectra similarity, value out of 1000. [2] $LRI_{cal.}$: experimentally determined linear retention indices. [3] $LRI_{lit.}$: linear retention indices reported from literature (65, 66). [4] Identification confirmed by authentic standard. [5] Compounds quantified in injection with 5 min HS-SPME sampling. [6] Compounds quantified in injection with 30 min HS-SPME sampling [7] Different superscript letters indicate significant differences ($p<0.05$). [8] Values are peak areas relative to internal standard

For semi-quantification purposes, peak area ratios of the identified compounds were calculated relative to 2 pentanone, the internal standard. This approach allowed relative quantification of compounds and consequently allowed comparison between the different treatments. To ensure the quality of peak integration, the peak table obtained from the automatic peak detection algorithm of the ChromaTOF software was manually re-integrated. The biggest problem with quantitation in GC×GC is the correct assignment of individual peak "slices" to a given compound. Random fluctuations in the modulation process might cause small shifts in the second dimension retention times, which might trigger the software to assume that the peak had finished eluting and to integrate subsequent slices as separate peak(s). Manual integration involved careful assignment of each individual slice to a peak based on its retention time and mass spectrum. The actual second dimension peaks were integrated using automated algorithms. This step was necessitated by the fact that very high relative standard deviations (RSDs) between repeat injections were obtained when only automated integration was applied. High RSDs would have rendered quantitative comparison of different wine samples using statistical methods impossible. In spite of re-integration, accurate quantification of the remaining compounds was not possible, in part due to tailing in the second dimension, which negatively affected the standard deviations.

Compared to previous studies utilizing 1-D GC on the same wines (42, 43), GC×GC-TOF MS offered several benefits. First, a relatively wide range of compounds could be identified and/or quantified accurately using HS-SPME extraction. For 1-D GC, liquid-liquid extraction in combination with FID detection may be used for the analysis of major compounds such as esters, alcohols, acids and fatty acids, whereas HS-SPME with MS detection in selected ion monitoring (SIM) mode is required to quantify selected carbonyls in the same wines (42, 43). All these compounds, and a significant number of additional volatiles, were successfully analysed in a single GC×GC-TOF MS analysis in this study. Secondly, the increased resolving power of GC×GC combined with the power of deconvolution of the TOF-MS mass spectra allowed for the identification, and in some instances quantification, of a much larger number of compounds in a single analysis. For example, more minor esters and compounds with terpenoid character were identified in the current study. Also, high-quality quantitative data could be obtained for more compounds in a single analysis, although this came at the price of much more intensive data analysis. Finally, the inherent sensitivity and wide dynamic range of GC×GC-TOF MS allowed the identification of major as well as minor compounds in a single analysis. For example, in the current study numerous compounds present at low levels (□g/L and lower) in wine were successfully identified. These include some of the terpenoids and methoxypyrazines, compounds for which analysis by 1-D GC often requires dedicated sample preparation and selective detection techniques.

All of these benefits allow us to report more qualitative and quantitative data in a single study, and therefore provided a significant step forward in studying in detail the chemical changes resulting from MLF using different starter cultures, as discussed in the following section.

4.3.2 Statistical analysis

4.3.2.1 Analysis of variance

Comparison of means by ANOVA and LSD testing showed significant differences in the levels of 43 out of 60 quantified compounds (Table 1) between the four treatments (the control and three MLF wines). As one biological repeat of the wines fermented with starter culture C was identified as an outlier in initial PCA and ANOVA analysis, both injections of this repeat were excluded from further data analysis.

For all three starter cultures, most of the 30 quantified esters increased after MLF. Levels of methyl hexanoate (19; numbers refer to compounds in Table 1), methyl octanoate (39) and ethyl phenylacetate (44) though tended to decrease following MLF, whereas 2 phenylethyl acetate (45), ethyl heptanoate (38) and isoamyl formate (8) showed no significant difference compared to the control wines. The general increase in the levels of esters following MLF agrees with the results of other authors (6, 14, 16, 43, 56), albeit some esters behaved differently (for instance, Ugliano and Moio (6) reported an increase in the levels of 2 phenylethyl acetate (45) and Bartowsky et al. (56) reported a general decrease of acetates).

Except for diacetyl (69), which originates from the citric acid metabolism of LAB (2), none of the carbonyl compounds increased following MLF for the wines studied here. All three LAB strains showed an increase in diacetyl (69), with starter culture O producing the highest levels of this compound. Diacetyl is responsible for the typical buttery flavour associated with MLF, as reported by numerous authors (1, 5). The other diketone, 2,3 pentanedione (71), decreased significantly in the MLF wines produced by all 3 starter cultures. Furthermore, benzeneacetaldehyde (88), 2 butanone (70), hexanal (85) and 2 heptanone (75) were also found to decrease significantly following MLF. A decrease of acetaldehyde, 2-methyl 1 butanal and 3 methyl 1 butanal has been reported in Chancellor wines following MLF (15). A decrease of acetaldehyde was also reported by others (13). On the other hand, the concentrations of 11 aldehydes in Syrah and Pinotage wines analysed by Malherbe (43) using HS-SPME-GC-MS did not show any significant differences as a result of MLF. Starter culture V seemed to differ in its metabolic profile, since the concentrations of 3 penten 2 one (72) and isobutanal (81) decreased significantly compared to the control wines, as well as to those produced with starter cultures O and C. Based on the ability of dairy Leuconostoc

species to reduce propanal to propanol, Liu (57) hypothesised that wine LAB are similarly able to reduce aldehydes to alcohols. The significant decrease of isobutanal (81) observed for wines fermented with starter culture V could indicate such ability for this culture. In addition, the corresponding alcohol isobutanol (52) increased significantly less in wines produced with starter culture V compared to cultures O and C. The remaining quantified carbonyl compounds showed no significant differences between the control and the MLF wines. Although diacetyl is one of the most studied compounds related to MLF, the change of other carbonyl compounds has not received much attention in previous literature reports. Our findings could therefore contribute to the understanding of the impact of MLF on this potentially influential class of wine volatiles and aid in the interpretation of sensory data.

For the wines analyzed in this study no general conclusions could be drawn with regard to the changes of higher alcohols due to MLF. Three of the four alcohols which showed significant increase after MLF (1 butanol (53), 1 propanol (51) and isobutanol (52)) were also present at significantly lower levels in wines produced from starter culture V compared to the other MLF wines. This is once again indicative of metabolic differences between strain V and the other LAB strains used here.

Levels of four of the five quantified terpenoid compounds also significantly decreased for all three starter cultures following MLF. Decrease of this class of compounds has been reported due to MLF by other groups (7, 8).

In the interpretation of the data reported here, it should be noted that the analyses in this study were performed eight months after bottling due to practical constraints on instrumental availability, and therefore relative levels of volatile compounds were subjected to various reactions occurring naturally during wine ageing. For instance, the levels of esters generally increase during wine ageing, except for esters produced by yeast during alcoholic fermentation in higher concentrations than those predicted by the law of mass action. Consequently, levels of those esters, e.g. ethyl esters of fatty acids, decrease during aging (58). This aspect hampers comparison of semi-quantitative data reported here with literature data for MLF wines. Nevertheless, this dataset is consistent in the sense that all analyses were performed within one week, which allows accurate comparison between the wines produced with the LAB strains investigated here. Our results therefore accurately reflect the effects of MLF on differences in volatile compounds in wines of the same age.

4.3.2.2 Multivariate data analysis

Principal component analysis (PCA) was used to identify the volatile components responsible for differentiation between control and MLF wines, as well as between MLF wines produced with different LAB starter cultures. For reasons of simplification only quantified compounds showing high correlation with the first two principal components (correlation coefficients > 0.8) were used for the presentation of the PCA model (Figure 3). The PCA biplot presented in Figure 3 provides an overview of the correlations of compounds with each of the MLF samples and the control wines. Excellent grouping of all biological repeats and duplicate injections was obtained for each MLF sample as well as for the control. Separation of all samples with different treatments was obtained by the first two PCs that explained 90% of the variance in the sample set (PC1: 79% and PC2: 11%, respectively).

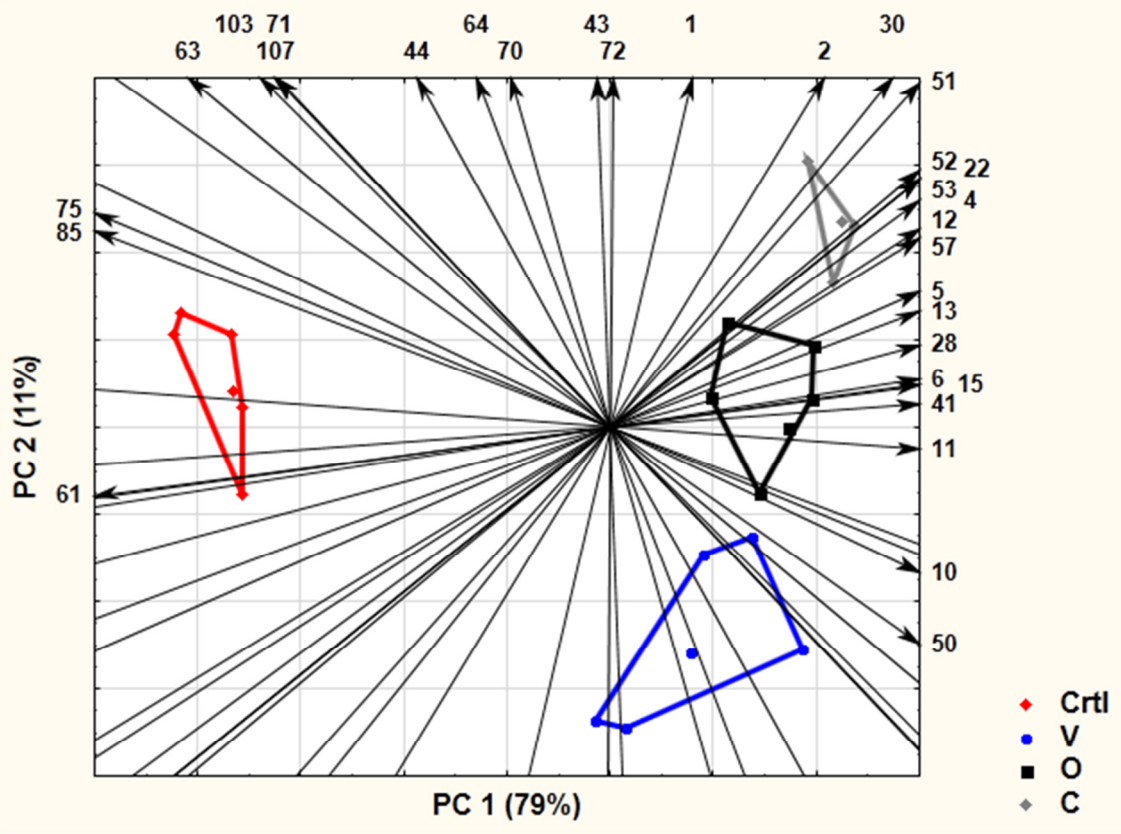

Figure 3: PCA biplot of volatiles quantified with high regression coefficients (R^2 > 0.8). Samples for each treatment are presented in the same color; their grouping is demonstrated with colored convex hulls. Vectors indicate different compounds, which are labeled corresponding to Table 1.

The control wines were separated from the MLF wines on PC1, whereas the variance between the different MLF wines was mainly explained by PC2. The control wines were positively correlated with 2 heptanone (75), hexanal (85), 1 hexanol (61), and to a lesser extent with 1 heptanol (63), 2,3 pentanedione (71), limonene (107), 6-methyl-5 hepten 2 one (103), ethyl phenylacetate (44), 1 octanol 3 ol (64), and 2 butanone (70) (the MLF wines showed negative correlation with these compounds). These compounds were largely responsible for the differentiation between the MLF and the control wines. Interestingly, hexanal (85) and 1 hexanol (61) are both associated with green odour descriptors (42, 43, 59), and a reduction in vegetative, green, grassy, herbaceous aromas following MLF has been reported previously (4, 23, 42, 43). A decrease in concentrations of compounds with terpenoid character after MLF (such as 6-methyl-5 hepten 2 one (103)) has been described previously (7, 8). Boido (7) assumed that these aroma compounds are able to form stable linkages with bacterial polysaccharides, therefore explaining their lower levels in MLF wines. On the other hand, according to D'Incecco et al. (27), partial metabolization of the liberated aglycon compounds by LAB may also be responsible for the lower concentrations of these compounds in MLF wines. Increased levels of glycoside-related volatiles, such as linalool, farnesol and damascenone (8) due to glycosidic activity during MLF, as reported by other groups (8, 27, 28), could not be confirmed in this work since only a few of these compounds were quantified.

All MLF wines correlated positively with isobutanol (52), 1 butanol (53), 1 propanol (51), amyl alcohol (57) and most of the esters. While the majority of wine esters originate from alcoholic fermentation by yeast, these results, in agreement with those of other researchers (6, 14, 16, 56), show that LAB can influence the relative concentrations of esters in wine. It is assumed that this is a result of bacterial esterase activity. Though less is known about esterase activity of wine-associated LAB, the same conclusion was drawn regarding the esterase activity of dairy associated lactic acid bacteria (60). In fact, a variety of enzymatic activities have been related to wine LAB (5, 60). Investigation of esterase activity of commercial MLF starter cultures was previously carried out by Matthews et al. (61). Esterase from O. oeni was first characterized by Sumby et al. (62), while the microbial modulation of esters in wine has recently been reviewed (63).

The MLF wines produced with different LAB strains were primarily differentiated according to PC 2. MLF wines from starter culture C and O were distinguished from those produced by starter culture V based on the levels of esters and some alcohols. These two cultures therefore seem to be alike regarding their metabolic activity in wine. Wines fermented with starter culture V differed in terms of negative correlation with the compounds 1 octen 3 ol (64), 2 butanone (70), methyl salicylate (43), 3 penten 2 one (72) and ethyl formate (1). The levels of these compounds, as well as diacetyl (69), isobutanal (81), isoamyl propanoate

(21), methyl acetate (2), propyl acetate (5), butyl acetate (12), isobutanol (52), 1 propanol (51) and 1 butanol (53), were significantly lower compared to wines produced with the other MLF starter cultures, once again indicating possible metabolic differences between this culture and the other LAB strains.

Higher alcohols are primarily derived from amino acid metabolism of yeast (64). Other groups, however, have also demonstrated that MLF, depending on the bacterial strain used, can have an impact on the concentration of higher alcohols (13, 14, 16, 24). Ugliano et al. (6) reported only small increases for several alcohols in their experiments when they studied changes of yeast-derived volatile compounds in Aglinanico wines.

Sensory studies of the wines analyzed in this study were performed five months after bottling (42, 43). The incidence of the odour descriptor “buttery” was significantly lower for starter culture V compared to starter cultures O and C, and did not show any significant difference compared to the control. Although the chemical analyses for the current study were performed three months later, it is likely that lower levels of diacetyl in wines fermented with starter culture V were responsible for this difference.

In conclusion, in this study GC×GC has successfully been applied for the improved separation of volatile compounds in Pinotage wines subjected to MLF. This has allowed the detailed investigation of the impact of different MLF starter cultures on the volatile composition of Pinotage red wine. The improved separation offered by GC×GC coupled with the use of deconvoluted mass spectra obtained by TOF-MS allowed the identification of a wide range of compounds in a single analysis, and enhanced the integrity of quantitative results through the reduction of the risk of co elutions.

The accurate relative quantification of 60 compounds provided useful new information regarding the changes in levels of individual compounds following MLF. With few exceptions, our findings were in accordance with published results regarding MLF. Moreover, the inherent advantages of GC×GC-TOF-MS in terms of improved resolution and sensitivity, combined with careful quantification, allowed the identification of a number of compounds showing significant differences as a function of MLF for the first time. These include several minor esters (1,4,5,12,13,15,18,19,21,22,34,35,39,43,48,49), 1 pentanol (57), the ketones 2 butanone (70), 3 penten 2 one (72) and 2 heptanone (75), the aldehydes isobutanal (81), hexanal (85) and phenylacetaldehyde (88), and 6 methyl 5 hepten 2 one (103). Most of these compounds cannot be easily identified and/or quantified by 1 D GC, due either to their low levels in wine, or to co elutions with other wine volatiles. The GC×GC TOF MS method reported here overcomes some of these problems, and as a result has contributed significantly to knowledge on the effect of MLF on Pinotage volatiles in particular.

While GC×GC is finding increasing application as a powerful screening tool for the identification of compounds in complex samples, our results also indicate the utility of the technique for quantitative comparison of wine samples. However, when using GC×GC-TOF MS, the polar nature of many wine volatiles and the concomitant poor peak shapes in the second dimension necessitate extensive manual intervention to ensure reliable quantitative data.

PCA and results from ANOVA and LSD testing indicated not only significant differences in the volatile composition between the control and MLF wines, but also the effect of metabolic differences between the MLF starter cultures studied here. Especially starter culture V showed significant differences compared to the starter cultures O and C, most markedly the lower amounts of diacetyl produced. Further investigation of the potential sensory contribution of the MLF-associated compounds reported here for the first time needs to be performed. More research on the biosynthesis pathways of lactic acid bacteria, wine ageing following MLF, the influence of grape cultivars on MLF, as well as the influence of winemaking practices on LAB are also required to fully elucidate the impact of MLF on wine aroma.

4.4 References

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5

Comparative study of two commercially available phases for stir bar sorptive extraction (SBSE) of wine volatiles combined with multi-dimensional gas chromatographic analysis

5.1 Introduction

Wine flavor is linked to a highly complex mixture of volatile compounds covering a wide range of physiochemical properties and concentrations. This makes the analysis of wine volatiles, as required for the investigation of wine aroma, challenging. Gas chromatography (GC) is the method of choice for the analysis of these volatile substances. Direct injection of wine samples onto the GC column is most often not recommended due to interfering matrix constituents, such as non-volatiles and water, resulting in poor chromatography and insufficient concentrations of the analytes of interest in the absence of a preconcentration step. Therefore sample preparation is a crucial step prior to GC analysis. However, in order to minimize sample alteration (e.g. due to degradation or loss of analytes), this pretreatment step should be as kept as simple as possible. The choice of sample preparation method depends on the properties of the analytes of interest. While sample preparation of major wine volatiles is rather straightforward (e.g. simple liquid-liquid extraction), the analysis of trace compounds, polar or unstable compounds is often laborious, expensive and can involve the use of harmful organic solvents.

To overcome some of these difficulties recent developments in sample preparation have largely focused on solvent free sorptive techniques such as solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE). SPME was developed by Arthur and Pawliszyn (*1*) in 1990, while SBSE was introduced by Baltussen and co-workers (*2*) in 1999. The principle of sorptive extraction can be compared to the partition process of liquid-liquid extraction, as PDMS is below its glass transition point at room temperature and therefore acts as a non-miscible liquid phase. Importantly, during sorption analytes do not temporarily bond with the material as occurs during the adsorption process (*3*). These techniques are easy to use and characterized by enhanced sensitivity compared to other sample preparation methods such as liquid-liquid extraction. The most commonly used polymer for sorptive extraction is polydimethylsiloxane (PDMS). This material is inert, shows excellent thermal stability (up to 320°C) as well as beneficial diffusion properties. Furthermore, PDMS degradation products all contain silicone and can therefore be easily differentiated from analytes of interest by mass spectrometry. This represents a significant benefit compared to other phases such as Tenax or Carbotrap 300, for which degradation products can interfere with the detection of target analytes, especially when thermal desorption (TD) is used (*3*). Thermal desorption of the SPME fiber is conveniently carried out within the hot GC injector port (*3, 4*), although liquid desorption with an organic solvent following sorptive extraction may also be used. Liquid desorption is usually used for the analysis of non-volatiles by liquid chromatography (LC) (*5*, *6*) or capillary electrophoreses (CE) (*7*, *8*), but can also be combined with GC (*9*).

SBSE was developed to overcome some drawbacks of SPME such as the adverse ratio between phase coating and matrix (the phase ratio), which results in reduced recoveries, especially relevant in trace analyses (*2*). However, commercial stir bars (Twister®, Gerstel, Mülheim an der Ruhr) are only available with PDMS phases, whereas SPME fibers are available with several coating types varying from PDMS to polar or mixed coatings. For example, the suitability of the following SPME fibers for the analysis of wine volatiles has been described in several studies: PDMS (*10-12*), carboxen/PDMS (CAR/PDMS) (*13*), PDMS/Divinylbenzene (PDMS/DVB) (*14*), DVB/CAR/PDMS (*14*), polyethylenglycol/DVB (PEG/DVB) (*15*), and polyacrylate (PA) (*16*, *17*). Note that the true sorption mechanism is lost for coatings which consist of copolymers (e.g. PDMS/DVB) and physical mixtures of PDMS with inorganic adsorbents (e.g. PDMS/CAR), as these no longer represent pure polymeric sorbents (*3*).

Pre-extraction derivatization reactions and *in situ* derivatization are often applied to overcome the unfavorable recoveries and chromatographic performance for polar compounds (*4*). However, the development of stir bar coatings with higher affinity for polar compounds would present a more convenient solution. In the last decade several in-house developed SBSE phases for GC and LC sample preparation have been reported, although no alternative to the PDMS Twister was commercially available until October 2010.

Most of these phases are only used in combination with liquid desorption (LD) due to low thermal stability. Stir bars with different groups introduced to PDMS, such as ß-cyclodextrin (ß-CD) (*18*, *19*), ß-CD/DVB (*20*) and poly(vinylalcohol) (*21*) have been prepared by sol-gel technology for use in combination with liquid desorption and GC or LC analysis. These phases demonstrated better recoveries for more polar analytes compared to PDMS. However, it was also reported that these coatings tend to crack, leading to gradual loss of phase over time. Another approach is the use of monolithic material. More polar compounds were successfully extracted by monolithic phases using several monomer mixtures, i. e. octyl methacrylate (MAOE)-ethylene dimethylacrylate (EDMA) (*22*), methacrylic acid stearyl ester (MASE)-EDMA (*23*), vinylpyridine (VP)-EDMA (*24*), vinylpyrrolidone (VPL)-DVB (*25*), vinylimidazole (VI)-DVB (*26*) and VP-EDMA (*27*). Liquid desorption and LC analysis were used for all analyses using these stir bar phases. In addition, a number of other coatings have been developed for the extraction of medium and low polarity compounds. Montes and co-workers (*28*) introduced polypropylene membranes suitable for immersion and headspace sampling in combination with liquid desorption and GC anlysis. Furthermore, Melo and co-workers (*29*) presented PDMS/polypyrrole (PPY) stir bars for the extraction of antidepressants followed by liquid desorption and LC analysis. The suitability of polyurethane (PU) foams as a more polar alternative to PDMS has also been reported (*30*). PU coatings in combination with liquid desorption and LC analysis have been successfully applied for the

analysis of Triazinic herbicides and acidic pharmaceuticals, both classes of highly polar compounds (*31*, *32*). Lastly, the development of stir bars based on alkyl-diol-silica (ADS) restricted access materials (RAM) (*33*) and molecular imprinted polymers (MIP) (*34*), both in combination with liquid desorption and LC analyis has been reported.

However, for the analysis of volatiles TD in combination with GC is preferred, since this provides better sensitivity and eliminates solvent interference. Recently developed thermally stable phases suitable for TD include the dual phase stir bars developed by Bicchi and co-workers (*35*) combine the concentration capabilities of two or more materials. In this approach a short PDMS tube of which both ends are closed by two magnetic stoppers provides space in its inner core for one or more further sampling materials. Phases used for this purpose included Carbopack (Supelco, Belefonte, Pennsylvania), Tenax GC (Buchem BV, Apeldoorn, The Netherlands), bisphenol-PDMS copolymer, and Carbopack coated with 5% of Carbowax (Supelco) (*36*). Other procedures for the production of in-house coatings have also been reported. Stir bars with a thermally stable porous hydroxy-terminated phase coating produced using sol-gel technology are suitable for TD-GC analysis of polar and apolar analytes (*37*). A thermally stable (up to 290°C) poly(phthalazine ether sulfone ketone) (PPESK) phase which allowed the usage of thermal desorption and GC analysis was developed by Guan and co-workers (*38*). Although the denser layer of the material hinders the transfer of analytes, better extraction of more polar compounds was obtained compared to PDMS stir bars. None of the alternative stir bar coatings discussed above have been used for the analysis of wine volatiles.

The company Gerstel recently introduced a new stir bar for SBSE with a polyethylenglycol-enriched (PEG) silicone phase called EG-Silicone Twister. According to Gerstel, the new stir bars have been successfully tested for whiskey volatiles, organic phosphorus pesticides and pesticides with relative low log $K_{o/w}$ values (< 3). For the analysis of whiskey volatiles the EG-Silicone Twister accumulates more species and higher amounts of phenols than the PDMS Twister. The PDMS basis of the EG-Silicone Twister also provides good affinity for non-polar analytes such as long chain ethyl esters. Similar results are expected for the analysis of volatile compounds in wine.

In this work the new EG-Silicone phase is compared to the conventional PDMS phase, to investigate differences in their extraction properties for volatile constituents in wine. Comprehensive two dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS) was used for this purpose during the first part of this study. In GC×GC, the combination of orthogonal separations using columns with different stationary phases results in much higher resolution (peak capacity) compared to conventional GC (*39-41*). Therefore the extra information resulting from enhanced separation power of GC×GC should allow the detailed investigation of the compounds extracted from the complex

wine matrix with each of these phases. While SPME in combination with GC×GC is, because of its convenience of being fully automated, well-established for the analyses of volatile wine constituents, to date only a few reports on the combination of SBSE and GC×GC-TOF-MS (*42-44*) have appeared, and to our knowledge this combination has never been applied to the analysis of wine volatiles.

During the second part of this study, heart-cutting two dimensional gas chromatography (GC-GC) in combination with nitrogen chemiluminescence detection (NCD) was used for the closer investigation of the extraction properties of the EG-Silicone Twister for three thiazoles (thiazole, 4-methylthiazole and 2,4-dimethylthiazole); these thiazole are thought to be involved in production of the ageing aroma of wine (*45-47*). SBSE-GC×GC-TOF-MS data showed the EG-Silicone phase to provide improved extraction of N-containing compounds. A previously developed method using liquid-liquid extraction for the analysis of nitrogen containing compounds including thiazoles in wine (*48*) was modified for SBSE-TD for this purpose. Heart-cutting was used to remove lower boiling nitrogen containing compounds which interfered with the target analytes (*48*). Contrary to GC×GC, in which the entire sample is introduced into the second dimension column by means of modulation, in heart-cutting GC-GC usually only one fraction from the first column is transferd into the second dimension column. The NCD is based on ozone-induced chemiluminescence and is not only a highly selective detector but is also sensitive and provides an equimolar response. The use of NCD for nitrogen containing compounds has been reported for several foodstuffs (*49-51*), including wine (*52-53*).

5.2 Material and methods

5.2.1 Chemicals and materials

For the determination of linear retention indices a series of C6 - C18 *n*-alkanes were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (ACS grade) was obtained from EMD Chemicals (Gibbstown, NJ, USA) and LS Labor Service GmbH (Griesheim, Germany). Standards of volatile compounds (Table 1) were purchased from Sigma-Aldrich, Fluka (Zwijndrecht, Netherlands), Riedel-de Haën (Steinheim, Germany), and Merck (Darmstadt, Germany). PDMS Twisters and EG-Silicone Twisters were obtained from Gerstel (Mülheim an der Ruhr, Germany). All Twisters had a phase volume of 32 µL.

For SBSE-TD-GC×GC-TOF-MS experiments a South African 2009 Sauvignon blanc and a 2009 Pinotage wine were analyzed. The alcohol content of the wines were 12.5 % and 11.9 %, respectively, and the pH's were of 3.5 and 3.7, respectively. For all SBSE-TD-GC-

-GC-NCD experiments a 2008 Riesling from the Rheingau region (Germany) was spiked with 200 µg/L of each thiazole. The wine had an alcohol content of 12.0 % (v/v) and a pH of 3.5.

5.2.2 Sample preparation

5.2.2.1 SBSE-TD-GC×GC-TOF-MS

For both stir bars, with PDMS and with EG silicone phase, the same extraction and desorption conditions were chosen. For SBSE 5 mL wine and 5 mL deionized water were transferred into a 22 mL headspace vial and the Twister introduced. The vial was capped immediately using a PTFE lined septum and aluminum cap. The mixture was stirred for one hour at 1000 rpm. After sampling the stir bar was removed, quickly washed with Milli-Q quality water, dryed using a lint free tissue and transferred to a glass desorption tube, which was immediately placed into the Thermal Desorption System (TDS) (Gerstel). Single analyses were performed. Furthermore, blank analysis of both, the PDMS and the EG Slilicone Twister were performed.

5.2.2.2 SBSE-TD-GC-GC-NCD

5.2.2.2.1 Headspace mode

After the pH of 20 mL of wine had been adjusted to different values (pH 12, pH 9, pH 6.5 and no adjustment pH 3.5,) with a 5 N sodium hydroxide solution, a 5 mL aliquot was transferred to a 22 mL headspace vial containing 1.5 g sodium chloride and a glass coated stir bar. The wine was then spiked with 200 µg/L of each thiazole by adding 100 µL of a standard solution containing 10 mg/L of each of the thiazoles in ethanol. The Twister was placed in an open glass insert for headspace sampling in the top of the vial, which was then directly capped using a PTFE-lined septum and aluminium cap. Extraction was performed with an agitation speed of 1000 rpm. Following the extraction, the Twister was removed from the vial, quickly washed with Milli-Q quality water and dried with a lint free tissue. It was then transferred to a thermal desorption tube, which was immediately placed into a Thermal Desorption Unit (TDU) (Gerstel). The CIS4 programmed temperature vapourization injector (PTV) was pre-cooled to -100°C and thermal desorption started without any delay. Extraction kinetics (1h, 2h and 3h) at a suitable pH were studied at room temperature and 40°C. Every analysis was additionally carried out with non-spiked wine to ensure that no artefacts were formed during the extraction. All analyses were performed in duplicate.

5.2.2.2.2 Immersion mode

For sampling in immersion mode wine samples were adjusted to pH 9 as described above. 10 mL of the wine was transferred into a 22 mL headspace vial containing 3 g sodium chloride. The wine was then spiked with 200 µg/L of each thiozole by adding 10 µL of a mixed standard solution containing 200 mg/L of each of the thiazoles in ethanol. The Twister was then put into the vial, which was directly capped using a PTFE-lined septum and aluminium cap. The subsequent procedure was identical to that described for the headspace mode (5.2.2.2.1). Extraction kinetics (1h and 2h) were studied at room temperature and an extraction for 1 h was performed without the addition of sodium chloride to study the salting out effect. Every analysis was additionally carried out with non-spiked wine to ensure that no artefacts were formed during the extraction. All analyses were performed in duplicate.

5.2.3 Thermal desorption

5.2.3.1 SBSE-TD-GC×GC-TOF-MS

Thermal desorption was performed in a thermal desorption system (TDS) connected to a CIS4 programmed temperature vaporizing (PTV) inlet (both Gerstel). After the desorption tube was manually placed into the TDS, desorption was performed as follows: 40°C for 0.5 min in “solvent vent” mode, ramped at 60°C/min to 220°C and held for 10 min (desorption flow 50 mL/min). The transfer temperature was set to 280°C. Analytes were trapped in the CIS at -100°C using liquid nitrogen. For injection onto the GC column the CIS was operated in split mode (100:1 for Sauvignon blanc and 20:1 for Pinotage), heated at 10°C/s to 280°C and kept for 10 min.

5.2.3.2 SBSE-TD-GC-GC-NCD

A thermal desorption unit (TDU) connected to a CIS4 programmed temperature vaporizing (PTV) inlet (both Gerstel) was used for thermal desorption. After the desorption tube was placed in the MultiPurpose Sampler (MPS) auto sampler (Gerstel), desorption was performed as follows: 40°C (delay time of 0.1 min) for 0.5 min in “solvent vent” mode, ramped at 120°C/min to 220°C held for 5 min (desorption flow: 50 mL/min). The transfer temperature was set to 280°C. Analytes were trapped in the CIS at -100°C using liquid nitrogen. For injection the CIS was heated at 12°C/s to 280°C and kept for 10 min. The CIS was operated in splitless mode for 2 min.

5.2.4 Chromatographic conditions

5.2.4.1 SBSE-TD-GC×GC-TOF-MS

An Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) equipped with a LECO thermal modulator (dual-stage quad-jet) and a secondary oven for the second dimension column was used. The GC was coupled to a Pegasus III time-of-flight mass spectrometer (TOF-MS) (both LECO Corp., St. Joseph, MI, USA). Separation was carried out in the first dimension on a 20 m Rxi-5Sil MS non-polar column (Restek, Belefonte, PA) with an internal diameter (i.d.) of 0.18 mm and a film thickness of 0.18 µm, which was coupled to a 2.0 m semi-polar Rtx 200 (RestekSGE, Belefonte, PA) second dimension column with an i.d. of 0.15 mm and a film thickness of 0.15 µm. A modulation period of 5 s was used with the cryogenic trap cooled to –196 °C using liquid nitrogen. The following oven temperature program was used for the primary oven: initial temperature 40 °C, kept for 5 min, ramped at 5 °C/min to 240 °C held for 5 min. The secondary oven was operated at a 20 °C offset from the primary oven. As carrier gas helium was used at a constant flow of 1.0 mL/min. The transfer line between the GC and the MS was maintained at 260 °C. Mass spectral acquisition was carried out in the mass range 35 - 450 amu at a rate of 100 spectra per second (ionization energy 70 eV). The ion source temperature was 200 °C and the detector voltage was set to -1750 V. The automatic peak detection algorithm of the ChromaTOF software (LECO Corp. version 2.22) was used for initial data processing. Positive identification was performed by analysis of authentic standards. The remainder of the peaks were tentatively identified based on mass spectral comparison with the NIST 08 library. First dimension linear retention indices (LRI) for each peak were automatically calculated by the ChromaTOF software using the retention times of a series of *n*-alkanes.

5.2.4.2 SBSE-TD-GC-GC-NCD

A dual oven system consisting of two Agilent 6890 GCs equipped with an Antek (Houston, USA) NCD Series 7090 were used. Separation was conducted in the first dimension on a 60 m DB-1 (J&W Scientific, Agilent Technologies) with 0.32 mm i.d. and a film thickness of 0.1 µm, and in the second dimension on a 60 m DB-WAX (J&W Scientific, Agilent Technologies) 0.32 mm i.d. and a film thickness of 0.25 µm. Column flow was set to 1.2 mL/min. The following oven program was used: for the primary oven; initial temperature 60 °C kept for 1 min, ramped at 10 °C/min to 230 °C, held for 36 min; and secondary oven: initial temperature 60 °C kept for 25 min, ramped at 5 °C/min to 180 °C, ramped at 10 °C/min to 230 °C and held for 8 min. Heart-cutting was performed using a Multi Column Switching Device (MCS) from Gerstel. To remove interfering low-boiling compounds, the heart-cut was

performed between 9 min and 21 min. The counter flow was set to 20 mL/min (helium). A cryotrapping device (CryoTrapSystem CTS1, Gerstel) was installed between the two columns, however, cryotrapping was not used in this experiment and the CTS1 was operated at 280°C. The NCD was operated at 950°C with a furnace vacuum of 140 Torr. The oxygen flow was at 10 mL/min and ozone flow was at 25 mL/min with vacuum in the detector chamber of 13 Torr. Chemiluminescence was detected in the range 600 to 900 nm.

5.3 Results and Discussion

Sorptive extraction techniques such as SPME and SBSE have found widespread application due to their advantageous extraction capability, easy handling and environmental friendliness. However, SBSE in particular is characterized by limited selectivity, as only PDMS phases were commercially available until recently. This is especially relevant for the analysis of wine volatiles, which comprise a wide range of compounds with different physiochemical properties, including numerous polar compounds such as alcohols, volatile acids and aldehydes. The goal of this work was therefore to study the extraction ability of a new SBSE phase, EG-Silicone, for volatile wine constituents. In order to perform a comparison of these phases, two approaches were followed. The first involved the analysis of a wide range of wine volatiles using GC×GC. Based on these results, in the second part a dedicated method for the analysis of thiazoles in wine following SBSE with the EG-Silicone-phase was developed for use in combination with GC-GC.

5.3.1 SBSE-TD-GC×GC-TOF-MS

GC×GC overcomes some of the limitations of one-dimensional GC, where separation occurs based on a single retention mechanism. The combination of two different retention mechanisms in GC×GC provide better resolution and a much higher peak capacity compared to conventional GC, which results in a significantly higher number of well resolved peaks for wine analysis. The column set up in this study, an Rxi-5sil (non-polar phase) in the first dimension and a semi-polar Rtx 200 column in the second dimension, proved to be suitable for the analysis of wine in other studies (*54*). Contour plots (total ion chromatogram, TIC) of the analysis of the Sauvignon blanc and the Pinotage wines are presented in Figures 1 and 2, respectively.

5.3.1.1 Performance of the chromatographic system

The combination of SBSE-TDS with GC×GC-TOF-MS was problematic for several reasons. These problems were largely associated with thermal desorption and cryotrapping in the PTV injector. All chromatograms showed poor peak shapes, with significant peak tailing in both dimensions. Poor peak shapes were likely caused by overloading of the chromatographic column. In GC×GC narrow bore columns are often used. As the loading a capacity of capillary column is dependent on its inner diameter, overloading can occur more frequently in GC×GC than in 1D GC, especially in the second dimension, where a narrow bore column is used for fast analysis. Furthermore, peak tailing in both dimensions (often called “bananagrams”) usually result when a compound is not injected into the first dimension column as a narrow band, but rather is introduced into the column over an extended period of time. The beginning of such a broadened band reaches the detector first at lowest oven temperature and therefore has the highest second dimension retention time. Subsequent fractions of the analyte elute from the second dimension column at higher temperatures and consequently have earlier second dimension retention times. Poor injection performance could be caused by several circumstances, such as a contaminated injector (e.g. particulate matter such as pieces of septum in the liner or graphite pieces from ferrules) This kind of peak tailing in GC×GC can be caused by any imperfectly deactivated surface, where polar compounds in particular will be retained and released at higher temperatures. For the experimental set up used here the cryofocussing unit was presumably the cause of this problem. Closer investigation was not possible as the instrument was only available for a limited period of time. However, despite these problems the retention times of compounds were reproducible in all samples and in the standard solutions.

A second problem with the injection system was associated with the fact that the EG-Silicone phase adsorbs more water due to its higher polarity. During cryofocusing water can cause blockage of the PTV, resulting in irreproducible injection. To prevent this problem, a solvent vent step (0.5 min at 40 °C) at the beginning of the thermal desorption was implemented (*55*). This allowed the water to evaporate. This setting led to loss of some low boiling analytes during the vent step as it was not systematically optimized. However, considering the limited availability of the instrument, this relatively long solvent vent step was chosen to reduce the risk of blockage.

Due to these problems the peak tables obtained from the automatic peak detection algorithm of the ChromaTOF software had to be manually re-integrated. Both the integration of each single peak slice in the second dimension chromatograms and the assignment of consecutive second dimension peaks were necessary. Although very time-consuming, accurate data analysis was possible. Using this approach, the GC×GC data allowed

comparison of peak volumes of a wide range of compounds with different physiochemical properties.

Sixtythree peaks in the Pinotage sample and 36 peaks in the Sauvignon blanc wine were tentatively identified by comparing deconvoluted mass spectra with the NIST 08 spectral library using ChromaTOF software, where a minimum match factor of 70% was used as criterion (Tables 1 and 2). An analyzed series of *n*-alkanes for the calculation of LRI could not be used, because calculated values for standard compounds did not match literature values. There was a general trend of the calculated values to be constantly approximately 50 units higher than the literature values. The solution of *n*-alkanes was directly injected into a desorption tube and not extracted using SBSE. Although the reason for the earlier elution of the *n*-alkanes is not clear, the divergent injection method used for wine samples and the *n*-alkane solution could be responsible for this phenomenon. The identity of a further 20 peaks in the Pinotage and 10 in the Sauvignon blanc was positively confirmed by means of injection of authentic standards. The majority of the identified compounds were esters, with smaller numbers of alcohols and acids. The remaining substances are phenolics, terpenes, sulfur and nitrogen containing compounds and others. A relatively large number of peaks (unknowns) could not be identified in both wines. However, none of the unknowns were present in the blank analyses. Note that a lower number of compounds identified in the Sauvignon blanc compared to the Pinotage wine is a result of a higher split ratio used for the former (100:1 for Sauvignon blanc and 20:1 for Pinotage), which was chosen to reduce overloading of the chromatographic system, which can affect peak shapes negatively.

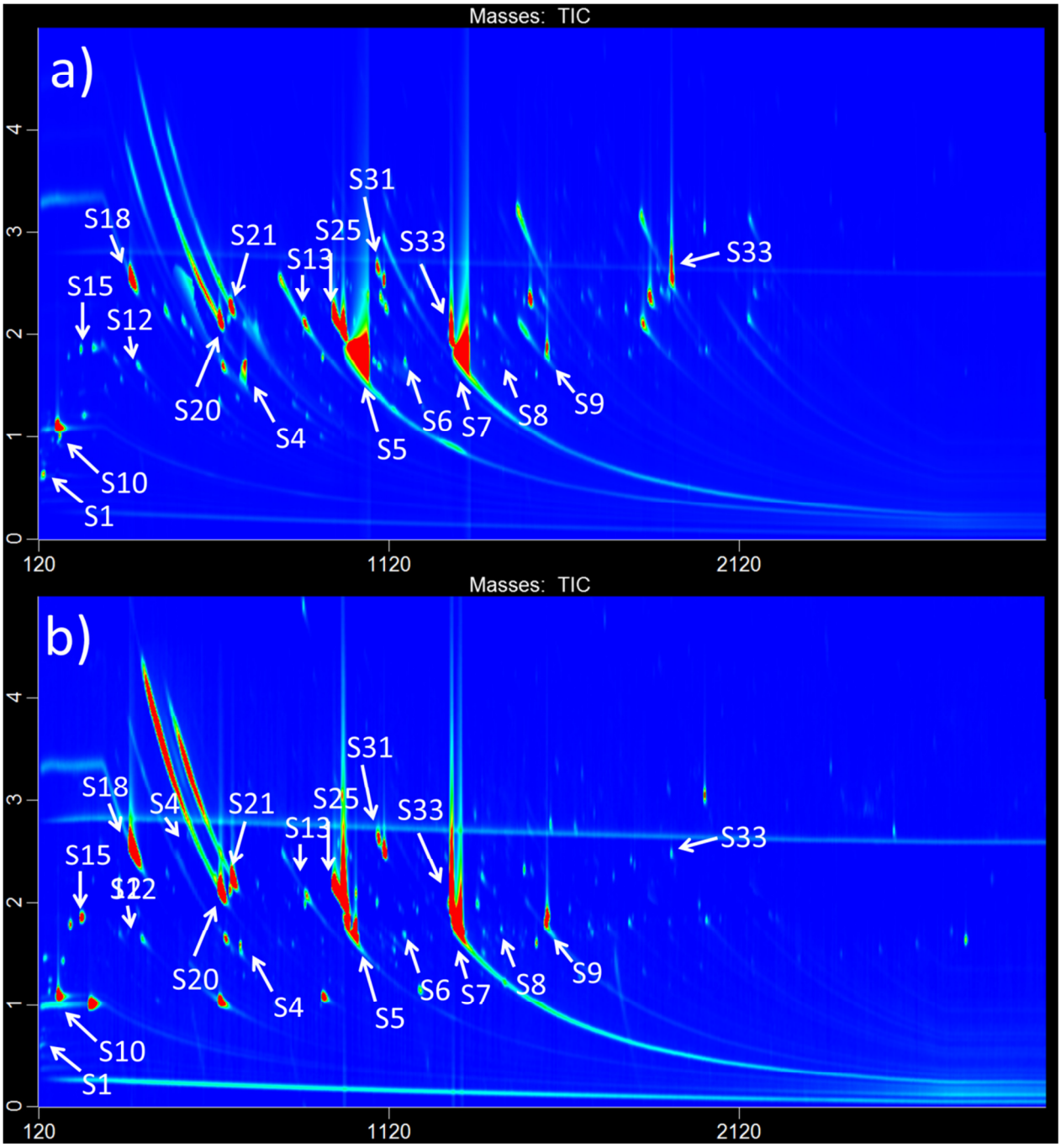

Figure 1: Contour plot of the analysis of a Sauvignon blanc wine using a) EG-Silicone and b) PDMS Twister for extraction followed by GCxGC-MS-TOF.

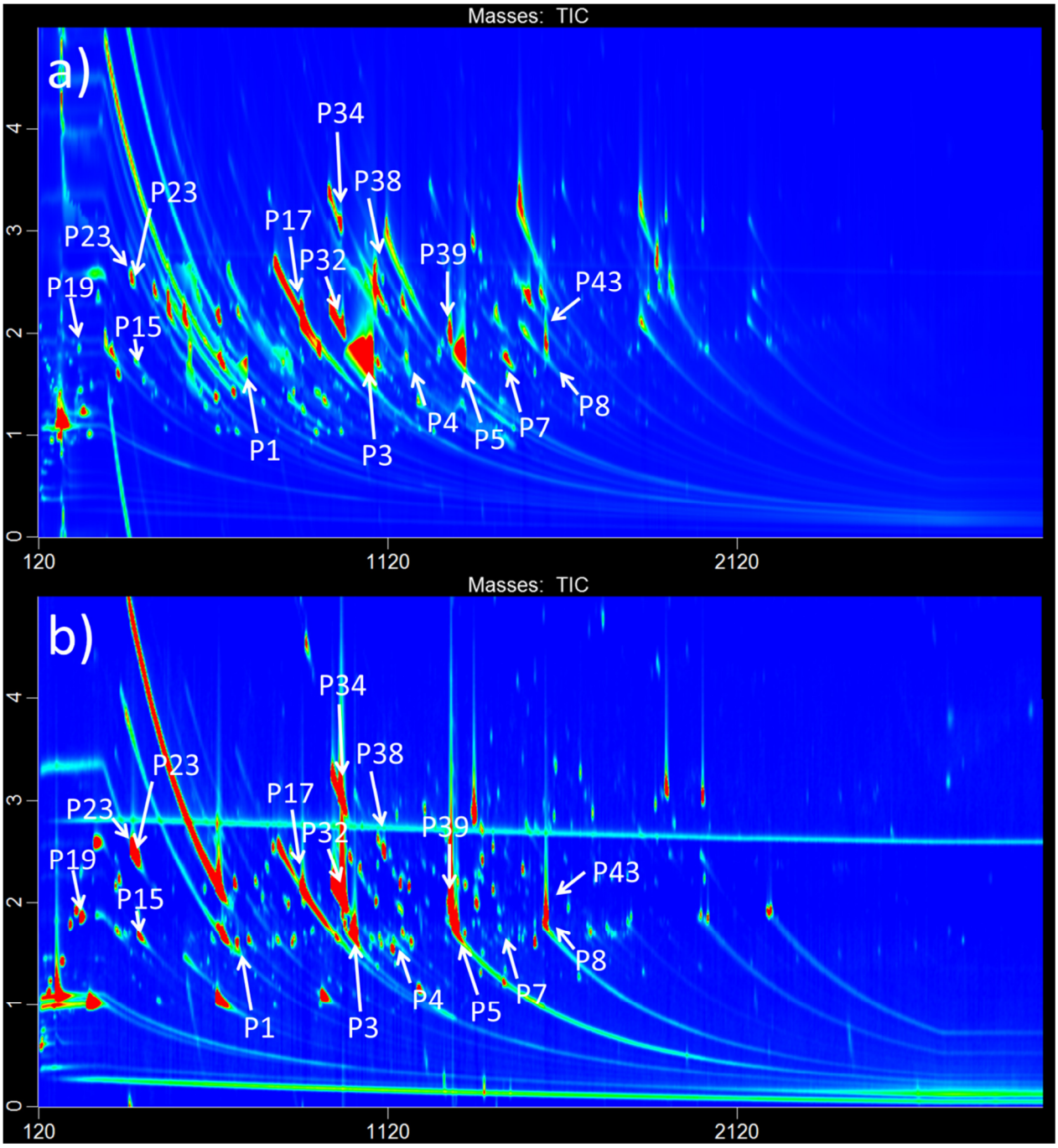

Figure 2: Contour plot of the analysis of a Pinotage wine using a) EG-Silicone and b) PDMS Twister for extraction followed by GCxGC-MS-TOF.

5.3.1.2 Comparison of the two phases for extraction of wine volatiles

To investigate differences in the extraction properties of the two stir bars, absolute peak areas (presented in Tables 1 and 2) of the analyses performed with the PDMS and EG-Silicone Twisters were compared. Note that replicate analyses could not be performed due to time constraints.

As expected, the more polar compounds in both wines showed higher affinity for the EG-Silicone phase. Especially the acids and alcohols were characterized by higher peak areas in the analysis using the EG-Silicone Twister. Aliphatic non-branched esters generally showed higher recoveries for the PDMS phase, whereas branched esters as well as the two unsaturated esters 2-hexenoic acid, ethyl ester (**P29,S23**) and 2-butenoic acid, ethyl ester (**P20**) showed only minor differences between the phases. Polar esters such as ethyl-S-lactate (**P26**), diethyl succinate (**P34, S27**) and esters containing aromatic groups (**P35, P38, P40, P41, P42, P44, P45, S31, S33, S36**) showed higher peak volumes when extracted with the EG-Silicone phase.

The EG-Silicone Twister also demonstrated better extraction for the remainder of the detected compounds belonging to various chemical groups. Most of these compounds have more polar functional groups, which could explain the higher affinity for this phase. Hetero atomic compounds such as the heterocyclic benzothiazole (**P47, S34**), showed significantly higher peak areas (Figure 3). Notably, methionol (**P46**) and indole (**P51, S36**) were only detected in the samples extracted with the EG-Silicone phase. In addition, much higher peak areas were obtained for phenolic compounds when the EG-Silicone Twister was used. 4-vinylguiacol was extracted in much higher levels with the EG-Silicone Twister. This compound is linked to “brett” off-flavor (*56*) produced by the spoilage yeast *Brettanomyces* when present in elevated levels. However, 4-vinylguiacol can also originate from oak wood extraction and occurs naturally at low levels in wine.

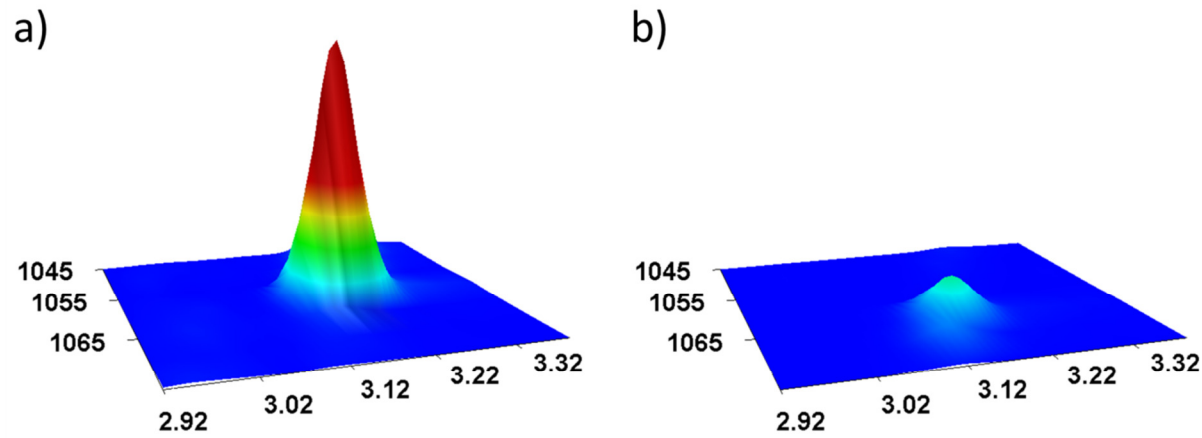

Figure 3: Three dimensional single ion chromatogram (135 m/z) of benzothiazole obtained using a) EG-Silicone and b) PDMS Twister. X- and y-axis are represented in seconds. Both chromatograms are on the same scale.

Despite the advantages of the EG-Silicone phase for the extraction of especially the more polar wine volatiles, the relative thermal instability of this phase was a significant disadvantage (especially compared to the conventional PDMS phase). The identification of PDMS degradation products with MS detection is straightforward due to the presence of characteristic siloxane mass fragments in the MS spectra. Molecules resulting from the breakdown of the EG of the dual phase Twister are often low molecular weight compounds containing oxygen, which makes their differentiation from wine volatile analytes difficult. This Twister releases the degradation products of both phases, which represents a significant drawback of the dual phase. This is illustrated by a chromatogram of a blank analysis with the EG-Silicone Twister, in Figure 4.

To conclude, SBSE in combination with GC×GC presents a sensitive chromatographic method, but requires extensive optimization before being considered as a viable method for the analysis of wine volatiles. Of the two commercially available Twister phases, the EG-Silicone phase showed higher extraction capability for polar volatiles. This phase, however, did not show advantage over the PDMS phase regarding major wine volatiles such as esters and acids, which are present at high concentrations in wine. The new EG-Silicone stir bar did, however, prove beneficial to the extraction of polar volatiles present at low concentrations in wine.

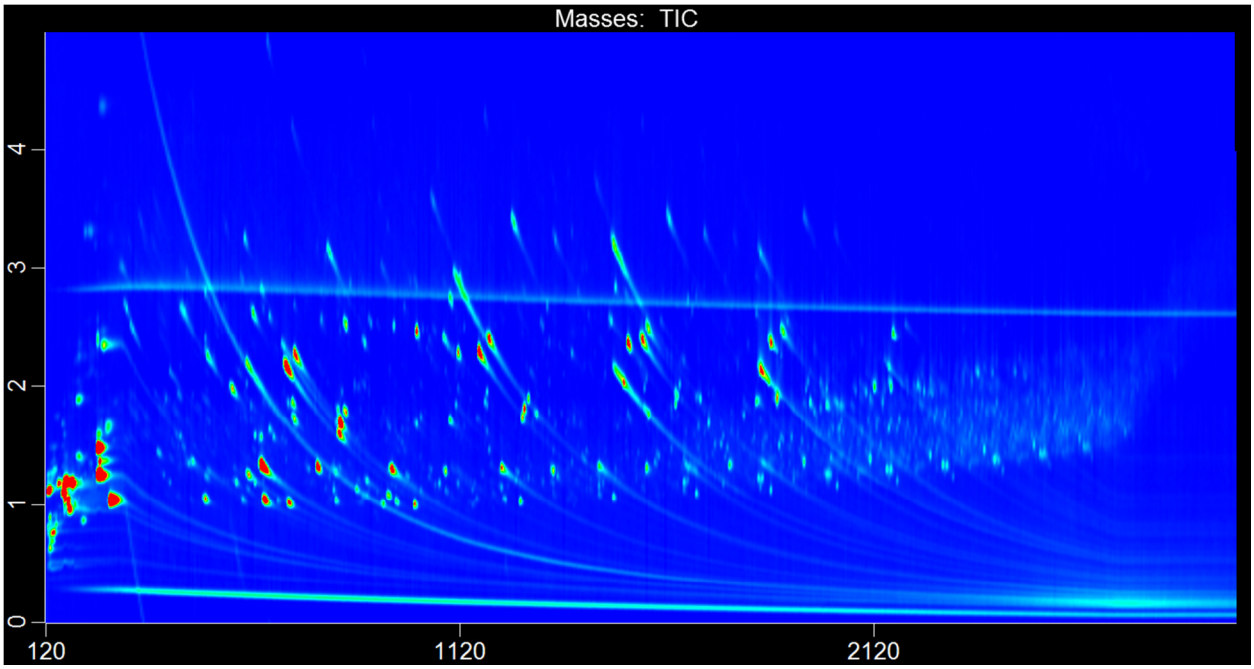

Figure 4: Contour plot of a blank analysis of the EG-Silicone Twister.

Table 1: List of compounds identified in a Pinotage wine sample by SBSE-TDS-GC×GC-TOFMS using PDMS and EG-Silicone [EG] Twisters.

No.	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]
	Acids					
P1	Hexanoic acid	EG	715	2,750	956	694039944
		PDMS	690	2,620	954	20385857
P2	Heptanoic acid	EG	870	2,670	871	4003077
		PDMS	not detected			
P3	Octanoic acid	EG	1075	2,890	833	1413721411
		PDMS	1025	2,830	930	275826329
P4	Nonanoic acid	EG	1175	2,780	895	189056516
		PDMS	1160	2,730	886	8737221
P5	Decanoic acid	EG	1335	2,910	818	2705878093
		PDMS	1315	2,860	927	316087823
P6	Geranic acid	EG	1315	2,910	866	78899015
		PDMS	1290	2,830	891	3681084
P7	Undecanoic acid	EG	1440	2,780	917	19953107
		PDMS	1440	2,750	906	2757523
P8	Dodecanoic acid	EG	1575	2,890	902	382020164
		PDMS	1570	2,840	898	123313071
P9	4-Hydroxybenzoic acid	EG	1605	2,790	869	8306738
		PDMS	not detected			
	Alcohols					
P10	1-Butanol, 3-methyl-[c]	EG	180	2,210	872	738165628
		PDMS	170	2,160	946	447975810
P11	1-Hexanol[c]	EG	395	2,750	950	217700812
		PDMS	405	2,700	950	34389636
P12	4-Methylpentanol	EG	325	2,840	934	299584810
		PDMS	345	2,730	908	10673738
P13	3-Methylpentanol	EG	350	2,790	839	7162273
		PDMS	365	2,710	925	3615551
P14	3-Hexen-1-ol[c]	EG	365	2,790	804	10961905
		PDMS	not detected			
P15	1-Hexanol[c]	EG	395	2,750	950	217700812
		PDMS	405	2,700	950	34389636
	Alcohols (continued)					
P16	1-Octanol[c]	EG	795	2,670	868	19200806
		PDMS	805	2,630	939	10677874
P17	Phenylethyl alcohol[c]	EG	870	3,280	912	396892892
		PDMS	875	3,170	963	333302431
	Esters					
P18	Acetic acid, 2-methyl propyl ester	EG	200	2,790	921	8962797
		PDMS	210	2,790	916	13975264
P19	Butanoic acid, ethyl ester (Ethyl butyrate)[c]	EG	235	2,860	944	57438901
		PDMS	245	2,870	936	64118578
P20	2-Butenoic acid, ethyl ester	EG	325	3,570	927	2266867
		PDMS	340	3,440	932	1555700
P21	Butanoic acid, 2-methyl-, ethyl ester	EG	325	3,270	943	5225003
		PDMS	340	3,170	945	6428978
P22	Butanoic acid, 3-methyl-, ethyl ester (Ethyl isovalerate)[c]	EG	335	3,340	891	10736486
		PDMS	350	3,240	883	11947735
P23	1-Butanol, 3-methyl-, acetate (Isoamyl acetate)[c]	EG	390	3,510	923	454489183
		PDMS	390	3,520	941	452499616
P24	1-Butanol, 2-methyl-, acetate[c]	EG	390	3,570	915	32030164
		PDMS	400	3,490	946	64070719
P25	Hexanoic acid, methyl ester (Methyl hexanoate)	EG	not detected			
		PDMS	505	3,130	899	648281
P26	Propanoic acid, 2-hydroxy-, ethyl ester, (S) (Ethyl-S-lactate)[c]	EG	550	2,850	834	594432661
		PDMS	540	2,470	882	18300026
P27	Hexanoic acid, ethyl ester (Ethyl hexanoate)[c]	EG	635	3,190	912	248968594
		PDMS	635	3,190	872	750376673
P28	Acetic acid, hexyl ester (Hexyl acetate)	EG	670	3,260	953	19276140
		PDMS	680	3,200	951	23863374
P29	2-Hexenoic acid, ethyl ester (Ethyl 2-hexenoate)	EG	730	3,280	891	4473660
		PDMS	740	3,220	928	4076378
P30	Heptanoic acid, ethyl ester (Ethyl heptanoate)	EG	825	3,040	853	5626708
		PDMS	835	2,990	931	6578697

No.	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]	No.	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]
	Esters (continued)							**Others**					
P31	Octanoic acid, methyl ester (Methyl octanoate)	EG PDMS	870 875	3,030 3,030	890 908	5458391 5367786	P46	Methionol	EG PDMS	645	3,140	920 not detected	9858126
P32	Octanoic acid, ethyl ester (Ethyl octanoate) [c]	EG PDMS	995 990	3,040 3,150	951 864	717519135 1848548306	P47	Benzothiazole	EG PDMS	1060 1065	3,190 3,160	901 820	9181405 1052091
P33	Benzoic acid, ethyl ester (Ethylbenzoate)	EG PDMS	970 965	3,450 3,420	915 934	463511 550406	P48	4-Vinylguiacol	EG PDMS	1200 1205	3,510 3,470	911 889	12462169 339433
P34	Butanedioic acid, diethyl ester (Diethyl succinate)	EG PDMS	955 980	4,360 4,100	964 957	1438279414 677953580	P49	2,3-Dihydrobenzofuran	EG PDMS	1090 1095	2,720 2,670	875 861	561946347 4682351
P35	Salicylic acid, methyl ester (Methyl salicylate)	EG PDMS	1000 1000	3,510 3,520	850 915	3647264 1434588	P50	3-Cyclohexene-1-methanol, α, α 4-trimethyl- (α-Terpineol) [c]	EG PDMS	1000 1000	2,820 2,820	841 889	8391621 3265507
P36	Hexanoic acid, 3-methylbutyl ester (Isopentyl hexanoate)	EG PDMS	1060 1085	3,160 3,010	911 902	15228075 12769834	P51	Indole	EG PDMS	1195	3,400	883 not detected	2561539
P37	Benzeneacetic acid, ethyl ester	EG PDMS	1065 1090	3,550 3,370	932 957	3735420 2364899	P52	Benzoic acid, 2,5-dihydroxy-, methyl ester	EG PDMS	1200	3,530	814 not detected	1150495
P38	Acetic acid, 2-phenyl ethyl ester	EG PDMS	1085 1110	3,690 3,490	934 948	207610649 65198903	P53	Eugenol [c]	EG PDMS	1260 1260	3,290 3,300	889 841	4359258 367944
P39	Decanoic acid, ethyl ester (Ethyl decanoate)	EG PDMS	1300 1300	3,020 3,000	840 800	482057032 610476801	P54	2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)- (Damascenone) [c]	EG PDMS	1290 1295	3,620 3,590	909 902	17156809 9111510
P40	Cinnamic acid, ethyl ester (Ethyl cinnamate)	EG PDMS	1420 1420	3,560 3,550	931 938	13900201 6566768	P55	4-hydroxybenzaldehyde	EG PDMS	1355	3,310	920 not detected	6821669
P41	Hexanoic acid, 2-phenyl ethyl ester	EG PDMS	1645 1645	3,240 3,250	864 883	1663089 690968	P56	5,9-Undecadien-2-one, 6,10-dimethyl-(Geranyl acetone)	EG PDMS	1385 1390	3,460 3,420	927 922	29731203 12968417
P42	Benzoic acid, benzyl ester (Benzyl benzoate)	EG PDMS	1810 1810	3,420 3,420	832 865	1576765 838860	P57	α-Farnesene [c]	EG PDMS	1455 1455	2,340 2,350	672 915	9121733 1923990
P43	Dodecanoic acid, ethyl ester (Ethyl dodecanoate)	EG PDMS	1570 1570	2,940 2,910	650 878	41922704 139954916	P58	Butylated hydroxytoluene [c]	EG PDMS	1460 1465	2,590 2,570	899 899	58414943 21660085
P44	Vanillic acid, ethyl ester (Ethyl vanillate)	EG PDMS	1595 1595	3,870 3,850	920 919	61072382 3715579	P59	Homovanillyl alcohol	EG PDMS	1535	3,950	899 not detected	1602294
P45	Succinic acid, 2-phenylethyl propyl ester	EG PDMS	1885 1885	3,980 3,960	893 880	13152812 5189714	P60	1H-2-Benzopyran-1-one, 3,4-dihydro-8-hydroxy-3-methyl- (Ochracin)	EG PDMS	1540 1545	0,260 0,210	926 907	3786588 744400

No.	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]
	Others (continued)					
P61	Nerolidol	EG	1540	2,620	937	52311951
		PDMS	1540	2,620	945	23945990
P62	Ethylparaben	EG	1560	3,240	952	10757058
		PDMS			not detected	
P63	Noreugenin	EG	2105	4,230	874	4902932
		PDMS			not detected	
	Unknowns					
P64	Unknown	EG	180	3,500		9833953
		PDMS			not detected	
P65	Unknown	EG	350	2,600		285913429
		PDMS			not detected	
P66	Unknown	EG	420	2,540		38151805
		PDMS			not detected	
P67	Unknown	EG	625	3,860		6726165
		PDMS	630	3,830		3323034
P68	Unknown	EG	840	2,630		12289056
		PDMS	845	2,600		4889300
P69	Unknown	EG	835	3,560		10622568
		PDMS	840	3,510		7793076
P70	Unknown	EG	885	0,630		21594541
		PDMS	890	0,540		21953090
P71	Unknown	EG	900	2,780		3830189
		PDMS			not detected	
P72	Unknown	EG	930	3,920		6842985
		PDMS			not detected	
P73	Unknown	EG	940	3,280		8813724
		PDMS			not detected	
P74	Unknown	EG	990	2,750		5007306
		PDMS			not detected	
P75	Unknown	EG	1055	3,300		2516696
		PDMS			not detected	
	Unknowns (continued)					
P76	Unknown	EG	1100	3,660		6455330
		PDMS	1105	3,630		514343
P77	Unknown	EG	1120	3,530		2925143
		PDMS	1125	3,490		2954330
P78	Unknown	EG	1110	2,630		20794003
		PDMS	1115	2,610		2248338
P79	Unknown	EG	1130	2,560		23816310
		PDMS	1135	2,540		22612332
P80	Unknown	EG	1200	3,530		1150495
		PDMS			not detected	
P81	Unknown	EG	1100	3,660		9380473
		PDMS	1125	3,490		3468673
P82	Unknown	EG	1060	1,730		14379548
		PDMS			not detected	
P83	Unknown	EG	1200	3,530		1150495
		PDMS			not detected	
P84	Unknown	EG	1245	3,350		6497580
		PDMS	1250	3,320		2458836
P85	Unknown	EG	1255	4,160		16739745
		PDMS			not detected	
P86	Unknown	EG	1370	3,000		47444923
		PDMS	1375	2,990		22501228
P87	Unknown	EG	1360	2,710		6739421
		PDMS			not detected	
P88	Unknown	PDMS	1385	3,750		9037641
		EG	1385	3,760		81776577
P89	Unknown	EG	1385	2,530		2648692
		PDMS	1390	2,510		1611759
P90	Unknown	EG	1390	4,310		7838533
		PDMS			not detected	
P91	Unknown	EG	1410	4,250		13402044
		PDMS			not detected	

No.	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]	No.	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]
	Unknowns (continued)							**Unknowns (continued)**					
P92	Unknown	EG	1515	2,990		2348252	P99	Unknown	EG	1910	3,840		2031344
		PDMS				not detected			PDMS	1910	3,830		216445
P93	Unknown	EG	1595	3,870		61072382	P100	Unknown	EG	1925	3,570		150912723
		PDMS	1595	3,850		3715579			PDMS	1925	3,480		1679918
P94	Unknown	EG	1620	3,500		62514830	P101	Unknown	EG	1935	3,380		18393838
		PDMS				not detected			PDMS				not detected
P95	Unknown	EG	1640	3,800		2972670	P102	Unknown	EG	1975	4,160		6495192
		PDMS	1645	3,760		998031			PDMS				not detected
P96	Unknown	EG	1700	2,720		12357076	P103	Unknown	EG	1990	4,280		5330820
		PDMS	1700	2,720		6556276			PDMS				not detected
P97	Unknown	EG	1790	4,110		1805567	P104	Unknown	EG	2005	3,820		12954953
		PDMS				not detected			PDMS				not detected
P98	Unknown	EG	1730	3,210		57740312							
		PDMS				not detected							

EG: EG-Silicone Twister. PDMS: PDMS Twister. [a] Mass spectra similarity, value out of 1000. [b] Absolute areas of deconvoluted Total Ion Current. [c] Identification confirmed by authentic standard.

Table 2: List of compounds identified in a Sauvignon blanc wine sample by SBSE-TDS-GC×GC-TOFMS using PDMS and EG-Silicone [EG] Twisters.

No.	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]	No.	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]
	Acids												
S1	Acetic acid	EG	135	1,650	979	63128764	S15	Butanoic acid, ethyl ester	EG	240	2,860	943	28107874
		PDMS	130	1,620	866	2905867		(Ethyl butyrate)[c]	PDMS	245	2,860	940	39573902
S2	3-Methylbutanoic acid	EG	425	2,490	891	4164604	S16	Butanoic acid, 2-methyl-,	EG	335	3,200	937	909315
		PDMS			not detected			ethyl ester	PDMS	345	3,130	914	1442020
S3	2-Methylbutanoic acid	EG	450	2,410	932	2978204	S17	Butanoic acid, 3-methyl-,	EG	345	3,270	879	1791047
		PDMS			not detected			ethyl ester (Ethyl isovalerate)	PDMS	350	3,230	904	2694724
S4	Hexanoic acid	EG	555	3,510	949	375985570	S18	1-Butanol, 3-methyl-, acetate	EG	385	3,560	897	84586647
		PDMS	520	3,450	888	5884023		(Isoamyl acetate)[c]	PDMS	395	3,530	893	91489573
S5	Octanoic Acid	EG	1030	2,920	933	5162873735	S19	Hexanoic acid, methyl ester	EG	495	3,190	892	829064
		PDMS	1025	2,810	931	213741024		(Methyl hexanoate)	PDMS	505	3,130	923	846780
S6	Nonanoic acid	EG	1165	2,750	908	36663711	S20	Hexanoic acid, ethyl ester	EG	635	3,170	795	359088415
		PDMS	1165	2,690	875	4318173		(Ethyl hexanoate)[c]	PDMS	640	3,140	942	1543859293
S7	Decanoic acid	EG	1325	2,900	931	3205740803	S21	Acetic acid, hexyl ester	EG	675	3,230	954	948944030
		PDMS	1325	2,910	932	1483418233		(Hexyl acetate)[c]	PDMS	675	3,230	958	745873336
S8	Undecanoic acid	EG	1440	2,770	914	6433741	S22	3-Hexen-1-ol, acetate, (Z)-	EG	660	3,150	929	40386782
		PDMS	1440	2,750	931	2520065			PDMS	665	3,130	928	17198527
S9	Dodecanoic acid	EG	1570	2,880	916	147820880	S23	2-Hexenoic acid, ethyl ester	EG	740	3,210	907	2677747
		PDMS	1570	2,850	918	96207415		(Ethyl 2-hexenoate)	PDMS	745	3,180	917	1711178
							S24	Octanoic acid, methyl ester	EG	875	3,020	911	6513884
	Alcohols							(Methyl octanoate)	PDMS	880	2,990	925	5578375
S10	3-Methylbutanol[c]	EG	180	2,090	905	240564421	S25	Octanoic acid, ethyl ester	EG	995	2,980	734	2103890929
		PDMS	175	2,110	948	187090715		(Ethyl octanoate)[c]	PDMS	960	3,270	756	910915060
S11	4-Methylpentanol	EG	340	2,750	920	16785492	S26	Nonanoic acid, 2-oxo-,	EG	975	3,590	815	3728756
		PDMS	350	2,700	918	2082023		methyl ester	PDMS	975	3,590	833	2124657
S12	1-Hexanol[c]	EG	405	2,690	955	46337999	S27	Butanedioic acid, diethyl ester	EG	985	4,030	967	14678887
		PDMS	415	2,650	959	10466583		(Diethyl succinate)	PDMS	985	4,040	963	9804816
S13	Phenylethyl alcohol[c]	EG	880	3,110	961	541744394	S28	Salicylic acid, methyl ester	EG	1000	3,510	955	5458314
		PDMS	885	3,070	964	40130848		(Methyl salicylate)	PDMS	1000	3,510	959	3270075
							S29	Benzeneacetic acid,	EG	1085	3,400	923	503918
	Esters							ethyl ester	PDMS	1090	3,370	936	373960
S14	Acetic acid, 2-methyl	EG	205	2,790	928	7158629	S30	Hexanoic acid, 3-methylbutyl ester	EG	1080	3,040	841	5168335
	propyl ester (Isobutylacetate)	PDMS	210	2,790	919	12116411		(Isopentyl hexanoate)	PDMS	1085	3,010	935	4275181

	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]
	Esters (continued)					
S31	Acetic acid, 2-phenyl ethyl ester	EG	1105	3,540	949	355797180
		PDMS	1105	3,530	947	187301989
S32	Decanoic acid, ethyl ester (Ethyl decanoate)	EG	1295	3,030	865	224098738
		PDMS	1300	3,050	902	293238424
S33	p-Hydroxycinnamic acid, ethyl ester	EG	1920	3,550	954	32196437
		PDMS	1925	3,490	950	4684161
	Others					
S34	Benzothiazole	EG	1060	3,190	836	490397
		PDMS	1065	3,150	826	295561
S35	2,3-Dihydrobenzofuran	EG	1075	2,750	871	57365581
		PDMS	1095	2,670	856	1232591
S36	Indole	EG	1195	3,390	917	1746345
		PDMS				
S37	4-Vinylguiacol	EG	1200	3,500	909	3114181
		PDMS	1205	3,470	846	622435
	Unknowns					
S38	Unknown	EG	1540	2,610		10038045
		PDMS	1540	2,610		8040071
S39	Unknown	EG	1385	3,450		7312804
		PDMS				not detected
S40	Unknown	EG	275	2,880		61227163
		PDMS				not detected
S41	Unknown	EG	290	3,590		4036427
		PDMS				not detected
S42	Unknown	EG	355	2,570		7974441
		PDMS				not detected
S43	Unknown	EG	880	3,590		8117082
		PDMS				not detected
S44	Unknown	EG	880	2,790		2744297
		PDMS				not detected
	Unknowns (continued)					
S45	Unknown	EG	895	2,770		1160189
		PDMS				not detected
S46	Unknown	EG	900	3,520		8082502
		PDMS				not detected
S47	Unknown	EG				
		PDMS	1150	2,950		1826771
S48	Unknown	EG	1190	3,570		3953541
		PDMS				not detected
S49	Unknown	EG	1200	3,530		3696281
		PDMS	1205	3,500		254058
S50	Unknown	EG	1215	3,440		6013598
		PDMS	1215	3,440		649206
S51	Unknown	EG	1370	3,000		10133079
		PDMS	1370	3,000		7255728
S52	Unknown	EG	1420	3,160		40834042
		PDMS				not detected
S53	Unknown	EG	1450	2,840		2290767
		PDMS				not detected
S54	Unknown	EG	1460	2,720		9188497
		PDMS				not detected
S55	Unknown	EG	1505	3,870		760926
		PDMS				not detected
S56	Unknown	EG	1605	3,510		3782560
		PDMS				not detected
S57	Unknown	EG	1610	3,520		1086802
		PDMS				not detected
S58	Unknown	EG	1590	2,830		8159799
		PDMS				not detected
S59	Unknown	EG	1560	3,160		2763218
		PDMS				not detected
S60	Unknown	EG	1795	3,260		27114362
		PDMS				not detected

	Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]		Compound	Twister phase	1D RT	2D RT	MS match[a]	Area[b]
	Unknowns (continued)							**Unknowns (continued)**					
S61	Unknown	EG	1720	3,220		14848160	S63	Unknown	EG	2765	2,630		1842262
		PDMS				not detected			PDMS	2765	2,640		8378521
S62	Unknown	EG	1920	3,380		24968494							
		PDMS				not detected							

EG: EG-Silicone Twister. PDMS: PDMS Twister. [a] Mass spectra similarity, value out of 1000. [b] Absolute peak areas of deconvoluted Total Ion Current. [c] Identification confirmed by authentic standard.

5.3.2 SBSE-TD-GC-GC-NCD

The SBSE-TD-GC×GC-TOF-MS results showed higher extraction capability using the EG-Silicone phase compared to the conventional PDMS phase for the nitrogen hetero cyclic compounds benzothiazole and indole in red and white wine. Based on these results closer investigation of the extraction properties of the EG-Silicone Twister for three thiazoles was conducted. Thiazole, 4-methylthiazole and 2,4-dimethylthiazole were chosen as they show increasing log $K_{O/W}$ values of 0.44, 0.97 and 2.09 (experimentally determined) (*57*). These thiazoles were previously reported in wine and fortified wine and are linked to the ageing aroma (*45, 46, 58*). It is assumed that they are formed in a Maillard-like reaction from dicarbonyl compounds and amino acids, although the mechanism of their formation under wine conditions is not yet fully understood (*45-47*).

Reported concentrations of these compounds in wine, sparkling wine an fortified wine range from 0.4 to 34 µg/L for thiazole, 0.2 to 11 µg/L for 2-methylthiazole, and 0.2 to 0.6 µg/L for 2,4-dimethylthiazole (*45*, *58*). Methods previously described for the analysis of thiazoles in wine were based on liquid-liquid extraction (*58, 45*). The drawbacks of liquid-liquid extraction such as labour intensity, use of harmful organic solvents and manual sample preparation are mostly overcome by sorptive extraction techniques such as SBSE. The main disadvantage of SBSE, that it exhibits low affinity for polar analytes using a PDMS phase, is overcome with the new EG-Silicone phase. This phase, due to the presence of a polar ethylene glycol phase, shows promise for the extraction of polar thiazoles in wine (as confirmed by GC×GC results presented previously) and was therefore used in this investigation.

A NCD was used to overcome the observed interference of EG degradation products when using MS detection. NCD reduces problems associated with co-elution by detecting only nitrogen compounds without halogen, phosphorous, hydrocarbon, or atmospheric nitrogen interferences (*59*). Neither in the blanks of the EG-Silicon nor in the blanks of the PDMS Twister were any peaks observed. The NCD is a selective detector for nitrogen compounds, but co-elution with other nitrogen compounds can still occur. Heart-cutting was used to remove low boiling nitrogen compounds, which interfered with the analytes of interest, as reported previously (*48*). Heart-cutting settings were adopted from a previous method (*48*) as follows: the fraction eluting between 9 min and 21 min from the apolar DB-1 first dimension column was sent to the polar DB-WAX second dimension column. The CTS1 was constantly operated at 280°C, as cryofocussing of the analytes prior to injection into the second column did not improve the second dimension separation. Furthermore, solvent vent settings were carefully optimized. When operating the TDU in solvent vent mode a delay time prior to solvent venting is usually programmed to provide a time window for the equilibration of the desorption flow and the venting temperature. A reduction of this delay time from 0.5 min to

0.1 min resulted in ~ 5-10 % increase of peak areas, while the increase was higher for thiazole compared to the other two compounds. However, the solvent vent step of 0.5 min at 40℃ was used according to instructions of the manufacturer Gerstel, to prevent injection complications that might occur due to blockage of the PTV. Following the adaptation of the chromatographic method (*48*) and sample introduction parameters, the SBSE extraction step was systematically optimized in both immersion and headspace modes. Figure 5 shows a chromatogram obtained for the analysis of the three target compounds in spiked wine.

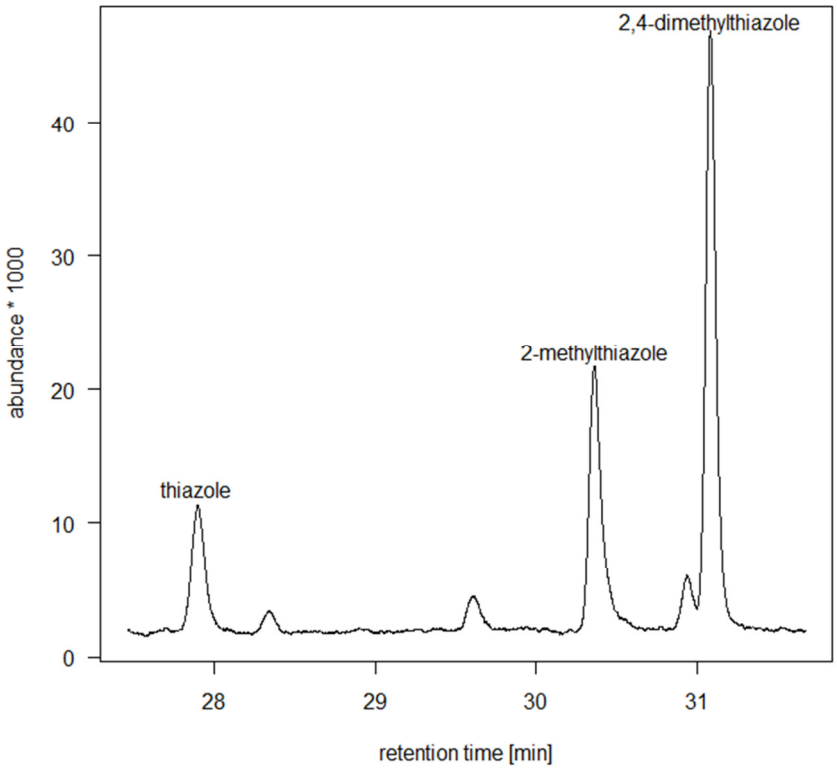

Figure 5: Second dimension chromatogram obtained for the EG-Silicone SBSE extraction of thiazole, 2-methylthiazole and 2,4-dimethylthizole in wine spiked with 200 µg/L of each analyte. Headspace extraction was performed at pH 12 for 3 h at room temperature with addition of 1.5 g sodium chloride.

5.3.2.1 Headspace mode

The effect of the following pH's on the headspace extraction of 5 mL wine spiked with 200 µg/L of each analyte was examined: pH 3.5 (no adjustment), pH 6.5, pH 9 and pH 12. Adjustment of pH was carried out using a 5N sodium hydroxide solution. Headspace sampling was performed for 1 h at an agitation speed of 1000 rpm at room temperature with

addition of 1.5 g sodium chloride. To ensure that no artefacts were formed during the extraction under these conditions, every analysis was additionally carried out with non-spiked wine. Furthermore, duplicate analyses were performed. The results are summarized in Figure 6. The pK_as of thiazole, 2-methylthiazole, and 2,4-dimethylthiazole are 2.52, 3.42 and 3.8, respectively (*60*). Protonation of the N-atom therefore occurs at low pHs, while at higher pHs the degree of protonation decreases, resulting in higher concentrations of the compounds in the headspace. The highest recoveries for all three thiazoles were obtained at pH 9. This is somewhat surprising, as at pH 6.5 complete deprotonation is already expected. Interestingly, at pH 12 peak areas for 2-methylthiazole and 2,4-dimethylthiazole decreased slightly compared to values at pH 9, although the reason for this is not clear. However other authors have used pH 12 during liquid-liquid extraction of thiazoles from foodstuffs (*61*). The increase in peak areas as a function of increasing pH was, as could be expected, more pronounced for the least polar compound, 2,4-dimethylthiazole. From pH 3.5 to pH 9 peak areas increased ~ 2.5× for thiazole, ~ 10× for 2-methylthiazole, and ~ 14× for 2,4-dimethylthiazole.

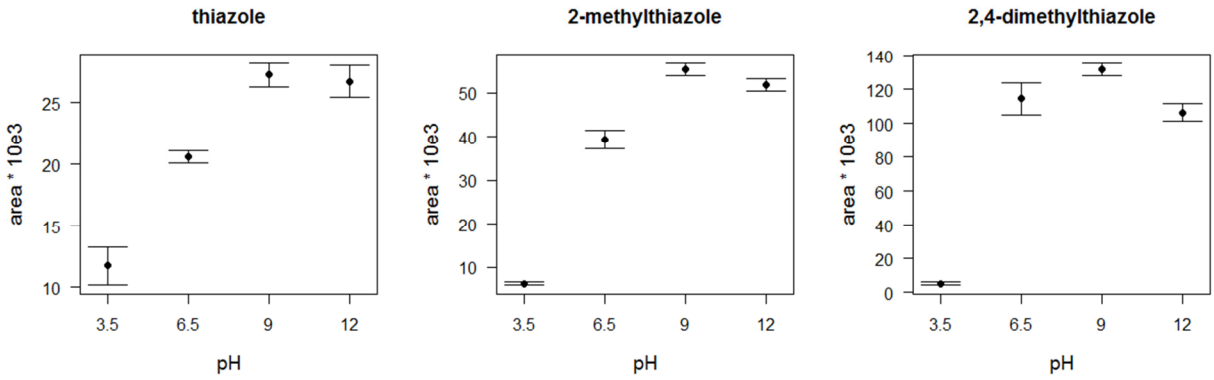

Figure 6: Peak areas for thiazole, 2-methylthiazole, and 2,4-methylthiazole as a function of pH (3.5, 6.5, 9, 12). Headspace extraction of 5 mL spiked wine (200 µg/L of each thiazole) using EG-Silicone Twisters for 1 h at room temperature with addition of 1.5 g sodium chloride. Mean values of duplicate injections are presented. Error bars represent minimum and maximum values.

Following pH optimisation, the extraction kinetics of the three thiazoles for the PDMS and the EG-Silicone Twisters were compared at room temperature. Extraction times of 1 h, 2 h, and 3 h were examined (Figure 7). Comparison of the recoveries of the two Twister phases confirms that all three compounds showed much higher affinity for the EG-Silicone phase. The polar portion of the dual phase showed the biggest contribution for the extraction of thiazole (lowest log $K_{O/W}$ of 0.44), which was not detected by extraction with the PDMS

Twister. Peak areas for 2-methylthiazole and 2,4-dimethylthiazole were ~ 4× and ~ 2×, respectively, higher for the EG phase compared to the PDMS Twister. 2-Methylthiazole was detected in very low concentrations following establishment of equilibrium (after 2 h) when extracted with the PDMS Twister. Also for this compound, the relatively low log $K_{O/W}$ (0.97) results in low extraction efficiency using this phase. Much higher recoveries were obtained when equilibrium was reached after 2 h for the extraction with the EG-Silicone Twister. Furthermore, peak areas for 2,4-dimethylthiazole (highest log $K_{O/W}$ of 2.09) differed only slightly for an extraction time of 1 h between the two phases, but the EG phase showed higher recoveries for longer times. Interestingly, equilibrium using the PDMS phase was reached after 2 h, whereas equilibrium on the EG-Silicone phase was not yet reached after 3 h. The fact that peak areas from the extraction with the EG-Silicone phase were only ~ 2-3× higher compared to the PDMS phase led to the assumption that both parts of the dual phase contribute significantly to the recovery of this compound. It is, therefore, evident that the discrepancy between extraction efficiency for the EG phase compared to PDMS increases with a decrease in log $K_{O/W}$.

The impact of temperature on the extraction kinetics of the three compounds for the EG-Silicone phase was also investigated. For this purpose extractions were performed at 40℃ for 1 h, 2 h, and 3 h. An extraction of non-spiked wine at 40℃ for 3 h using the EG-Silicone Twister was also carried out to ensure that no artefacts were formed during sampling at this temperature. The increase in temperature led to faster establishment of equilibrium for all three compounds. The equilibrium for thiazole was reached after 2 h, where for 2-methylthiazole and 2,4-dimethylthiazole an extraction time of 1 h was sufficient. Interestingly, the recoveries decreased steadily for 2,4-dimethylthiazole for extraction times longer than 1 h.

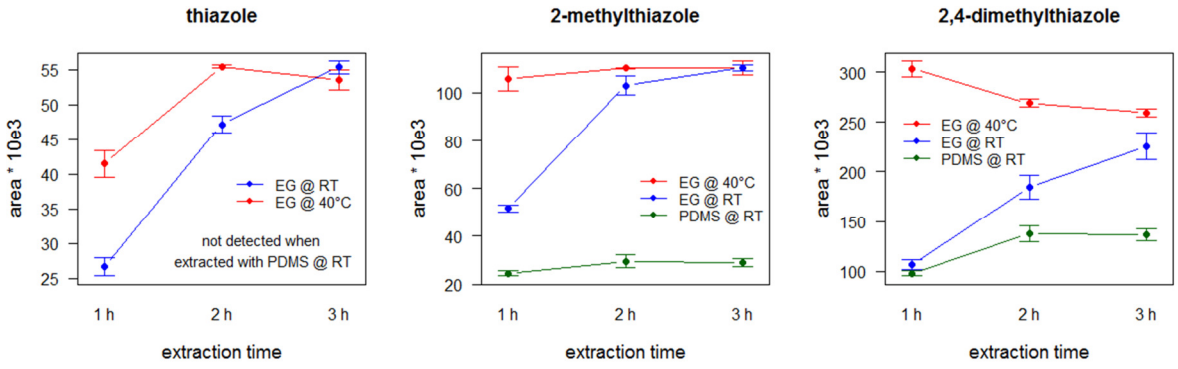

Figure 7: Extraction kinetics for thiazole, 2-methylthiazole, and 2,4-dimethylthiazole as a function of extraction time (1 h, 2 h, 3 h) and temperature (room temperature [RT], 40 °C) for the different Twister phases (EG-Silicone [EG], PDMS). Headspace extraction of 5 mL spiked wine (200 µg/L of each thiazole, pH 12). Mean values of duplicate injection are presented, whereas error bars represent minimum and maximum values.

5.3.2.2 Immersion mode

To compare the extraction ability of the EG-Silicone and PDMS Twisters in immersion mode to those of the headspace mode, wine pH was adjusted to 9 as established in headspace mode and the wine was spiked with 200 µg/L of each compound. According to the manufacturer (Gerstel) the pH range for the application of the EG-Silicone Twister in immersion mode is pH 3.5 - pH 10. The samples were stirred at 1000 rpm at room temperature. To study the effect of salt addition, extractions were carried out for 1 h at room temperature with the EG-Silicone Twister with and without the addition of 3 g sodium chloride. Additionally, to investigate the extraction kinetics in immersion mode extractions with both Twisters were carried out for 1 h and 2 h at room temperature with addition of 3 g sodium chloride. To ensure that no artefacts were formed at these conditions, the analysis with an extraction time of 1 h at room temperature (with salt) using the EG-Silicone Twister was additionally carried out with non-spiked wine, where no peaks for the target analytes where detected. All analyses were performed in duplicate. Results are summarized in Figure 8.

The addition of salt led to a significant increase of the peak areas for all three compounds. For both Twisters equilibrium was already reached after 1 h extraction at room temperature in immersion mode, in contrast to headspace mode. Analogous to the sampling in headspace mode the difference between the extraction efficiency for the EG-Silicone phase compared to the PDMS phase increases with the decrease in log $K_{O/W}$. This clearly suggests the substantial contribution of the EG phase of the dual phase twister to the extraction of these hetero-atomic compounds.

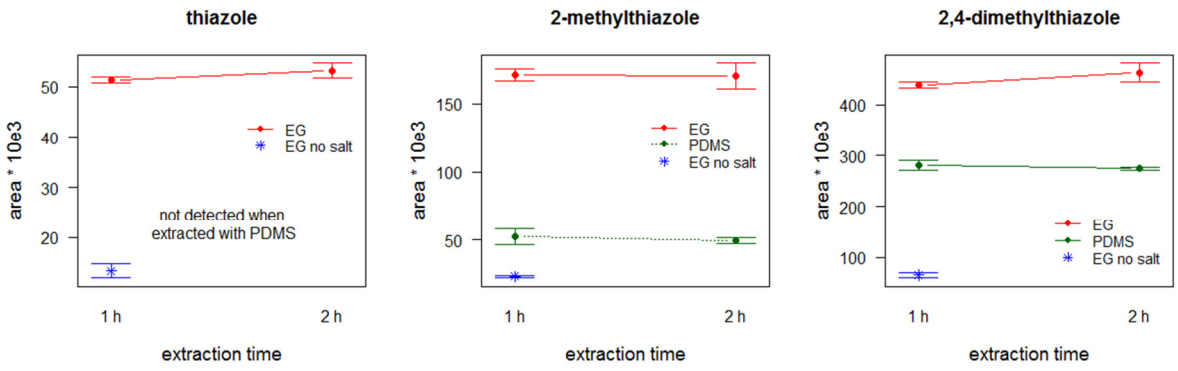

Figure 8: Peak areas for thiazole, 2-methylthiazole, and 2,4-methylthiazole as a function of extraction time for both phases and as a function of salt addition for the EG-Silicone phase. Immersion extraction of 10 mL spiked wine (200 µg/L of each thiazole, pH 9) at room temperature with 3 g sodium chloride. Mean values of duplicate injection are presented, whereas error bars represent minimum and maximum values.

In conclusion, extraction in immersion mode is compared to headspace sampling not only faster, but also more efficient for 2-methylthiazole and 2,4-methylthiazole, whereas recoveries for thiazole did not differ between the two extraction modes under optimal conditions.

The limit of detection for each thiazole was estimated from the signal obtained from the analysis of spiked wine (200 µg/L) under optimized conditions (immersion sampling for 1 h at room temperature at pH 9 with addition of 3 g sodium chloride). Limits of detection at signal-to-noise ratios of 3:1 were 25 µg/L for thiazole, 8 µg/L for 2-methylthiazole, and 4 µg/L for 2,4-dimethylthiazole. Considering reported concentrations of these compounds in wine, sparkling wine an fortified wine vary between 0.4 to 34 µg/L for thiazole, 0.2 to 11 µg/L for 2-methylthiazole, and 0.2 to 0.6 µg/L for 2,4-dimethylthiazole (*45*, *58*) the current SBSE method is not sensitive enough for the analysis of these compounds in most wines.

5.4 Summary and conclusions

In the first part of this study, the application of SBSE-TDS-GC×GC for the analysis of wine volatiles using two different stir bar phases was investigated. These analyses demonstrated several problems during this study. Poor peak shape, particularly extensive peak tailing, resulted from overloading and possible sorption and or adsorption of the analytes on active sites in the chromatographic system, which are then released at higher temperatures. Furthermore, during cryotrapping of the thermally desorbed analytes from the EG-Silicone Twister blocking of the PTV injector can occur due to the presence of water extracted by the

EG phase. Further optimization of the thermal desorption and solvent vent settings, and a detailed investigation of possible effects of non-perfectly de-activated parts in the GC system are therefore required. However, this could not be performed in the timeframe of this study due to limited instrument availability. It should be mentioned that these difficulties are not inherent to SBSE-TDS in combination with GC×GC-TOF-MS, as this combination has previously previously been applied successfully (*42-44*).

In sorptive extraction the partition coefficients of analytes between the PDMS phase and the aqueous phase, correlated to the octanol/water coefficient, governs equilibrium. The recovery of polar compounds (low $K_{O/W}$) is therefore relatively poor on the apolar PDMS phase. Favorable extraction capacity for both non-polar and polar compounds was presented for the double phase EG-silicone Twister. Compared to the conventional PDMS Twister, the EG-Silicone Twister also showed sufficient extraction performance for non-polar compounds. With increasing polarity of analytes, however, the Silicone-EG Twister provided better results. Furthermore, compounds with hetero-atoms or phenolic groups showed much higher affinity for the EG-Silicone phase. Since these are often trace level compounds, use of this phase for wine analysis may show promise. The EG-Silicone Twister is clearly a promising alternative for the extraction of compounds with low log $K_{O/W}$ values (< 3) in wine, such as some sulfur and nitrogen containing compounds. A major drawback of this phase is, however, the lack of thermal stability, which is especially important when using TD. To overcome the drawback of interfering peaks resulting from degradation products of EG phase, selective detectors such as chemiluminescence or pulsed flame photometric detection for sulfur and chemiluminescence or nitrogen phosphorus detectors for nitrogen would be useful.

In the second part of this study three thiazoles were chosen for closer investigation of the extraction properties of the EG-Silicone Twister for heterocyclic compounds in wine. The combination of EG-Silicone Twister with heart-cutting analysis and nitrogen selective detection (SBSE-TD-GC-GC-NCD) provided an alternative tool for the analysis of thiazoles in aqueous samples. Nitrogen selective detection was used to overcome problems associated with thermal instability of the EG phase, which causes presence of unwanted low molecular weight interfering breakdown products. The use of heart-cutting GC eliminated co-elution with low-boiling nitrogen compounds. Different extraction parameters for headspace and immersion mode were investigated. The comparison of the EG-Silicone Twister and the PDMS Twister showed much better extraction abilities for the EG-Silicone phase for all three thiazoles in both extraction modes. The extraction method did not affect the maximum yielded peak areas for thiazole, while higher recoveries were obtained for 2-methylthiazole and 2,4-dimethylthiazole in immersion mode. Furthermore, extraction was faster for all compounds when the Twister was immersed during sampling. In headspace mode, salt

addition extended extraction time while increased extraction temperature resulted in better recoveries for all compounds. 2,4-Dimetyhlthiazole showed different extraction behavior compared to the other two compounds during headspace sampling at 40 °C. The influence of the pH on the head space extraction as a function of pK_a of the compounds was also demonstrated.

For the optimized conditions, which are immersion sampling for 1 h at room temperature at pH 9 with addition of 3 g sodium chloride, the limits of detection (at signal-to-noise ratios of 3:1) were calculated as 25 µg/L for thiazole, 8 µg/L for 2-methylthiazole, and 4 µg/L for 2,4-dimethylthiazole. Therefore this method is not sufficiently sensitive for the analysis of the majority of wine samples, considering the typical concentration ranges of these compounds in wine (*45*, *58*) and other extraction methods would be preferable.

Considering the applicability of the different phases in SBSE it can be concluded that for untargeted screening of wine volatiles the PDMS phase is preferable because of its thermal stability which outweighs the less efficient extraction of more polar analytes. However, for targeted analysis of polar volatiles, especially when making use of selective detectors, the EG-Silicone phase provides a marked improvement.

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6

Investigation of the composition of volatile sulfur and selected nitrogen compounds of Pinotage wines fermented with different malolactic starter cultures

6.1 Introduction

The principle reason for malolactic fermentation (MLF) is to accomplish biological deacidification of wine. During MLF lactic acid bacteria (LAB), most commonly *Oenococcus Oeni*, convert the harsh-tasting L-malic acid into milder tasting L-lactic acid, resulting in enhanced biological stability and improved mouth feel of wine (*1-3*). During MLF LAB also produce volatile metabolites and modify aroma compounds and flavor precursors originating from grapes and alcoholic fermentation. Hence MLF also has an impact on wine aroma (*4*).

One of the most important and best investigated aroma compounds formed during MLF through citric acid metabolism is the diketone diacetyl (2,3-butanedione). Mainly described with “buttery” attributes, diacetyl can also contribute to “nutty” and “toasty” aromas at low concentrations (*3, 5*). Several reviews on the sensory impact and methods for diacetyl management in wine have been published (*2, 6, 7-9*). The impact of MLF on the concentrations of different wine volatiles such as esters (*5*), alcohols (*5*), volatile phenols (*10*), terpenoids (*11*, *12*) and sulfur compounds (*13*) have also been studied, albeit not as extensively as the role of diacetyl.

Sulfur and nitrogen containing compounds, however, are to a large extent neglected groups of compounds when it comes to the investigation of the aroma impact of MLF. The combination of the specific concentration of sulfur containing compounds, their aromatic characteristics and synergist–antagonist effects result in both positive or negative sensory impressions on wine aroma such as enhanced fruitiness and reductive off-flavors, respectively. Different chemical classes of sulfur-compounds are found in wine, including thiols, thioesters, sulphides, polysulphides and heterocyclic compounds. In terms of the gas chromatographic analysis, sulfur containing compounds are often for practical reasons categorized into compounds with low boiling points (< 90 °C) and high boiling points (> 90 °C) (*14*,*15*).

Wine and cheese associated LAB can metabolize sulfur-containing amino acids such as methionine. The degradation of this amino acid can lead to the formation of odor active volatile sulfur compounds (VSCs) such as hydrogen sulfide, methanethiol, dimethyl sulfide, dimethyldisulfide, methional, methionol and 3-methylthio propanoic acid (*16-20, 10*, *13*). A recently cloned and characterized cystathionine β/γ-lyase from *Oenococcus oeni* (*O. oeni)* oenological strains (*21*) was able to degrade sulfur containing amino acids such as homocysteine, methionine, cystathionine and cysteine. It was hypothesized that the degradation of these sulfur containing amino acids by *O. oeni* could contribute to the formation of VSCs in wine. Further investigation of the enzymatic activity under harsh wine conditions is, however, still necessary.

Increased concentration of VSCs can affect perceived wine quality negatively or positively. Whereas VSCs in high concentrations are often linked to sulfur related off-flavors (reductive notes, for example methionol), increased concentration of 3-methylthio- propanoic acid have a favorable impact on wine flavor (*13*). Some sulfur containing compounds are associated with sulfide off-flavors, such as rotten egg, cooked cabbage, cauliflower and burnt rubber. The most relevant compounds associated with ojectionable wine aroma are hydrogen sulfide, methanethiol (methylmercaptan), ethanethiol (ethylmercaptan), dimethyldisulfide, dimethyltrisulfide and thioesters (*22*). These off-flavors can be formed by chemical, photochemical, or thermal reactions during vinification and storage, but even more important are enzymatic reactions. The wine yeast *Saccharomyces cerevisiae* forms these compounds under nutrient deficiency via its nitrogen and sulfur metabolism. A shortage of assimilable nitrogen in the grapes in particular leads to a lack of nitrogen in the must, and therefore production of these compounds by yeast (*22*, *23*).

Some nitrogen compounds such as 2-aminoacetophenone (2-AAP), indole, skatole, and anthranilic acid esters are linked to atypical aging off-flavor in wine. Nutrient deficiency in the vineyard and water stress favors the formation of this off-flavor. The impact of MLF on the levels of these compounds is to the best of our knowledge not known.

Several GC methods for the detection and quantification of sulfur compounds in wine have been reported. GC coupled with mass spectrometry (MS) is often used in single ion monitoring (SIM) mode (*24-26*). However, co-elution of sulfur compounds with other wine constituents is problematic when using MS detection. Therefore, sulfur selective detectors with an equimolar response, such as the pulsed flame photometric detector (PFPD) or the sulfur chemiluminescence detector (SCD) are preferred for quantification purposes (*27*, *28*). These detectors show high selectivity for sulfur. Furthermore, due to the complex wine matrix, low concentrations and the high reactivity of sulfur compounds, special attention must be paid to sample preparation (*14*).

In previous work the effect of different LAB starter cultures on the volatile composition of Pinotage wines was investigated using GC-FID and GC-MS (*29*) and comprehensive two dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS) (*30*). While significant differences were observed in the volatile composition of wines produced with different LAB starter cultures, these data were focused both on levels of specific compounds (*29*) and also on the untargeted GC×GC analysis (*30*) of volatile compounds. However, none of these methods provided detailed information on the composition of sulfur compounds in experimental wines. The aim of this study therefore was to investigate changes in the concentrations of VSCs of the same Pinotage wines in order to determine whether the use of different starter cultures during MLF also affects the levels of these compounds. For this purpose two different GC methods were used for the quantitative

analysis of low boiling sulfur compounds (*31*) and for the simultaneous determination of higher boiling sulfur compounds and selected nitrogen compounds (*32*) in this study. For the analysis the of low boiling sulfur compounds headspace (HS) sampling together with PFPD detection was used, whereas for the simultaneous determination of higher boiling sulfur and nitrogen compounds solid supported liquid-liquid extraction (SLE) in combination with GC-MS and GC-SCD was applied.

6.2 Material and Methods

6.2.1 Wine samples

The Pinotage wines produced with four commercial starter cultures used in this work originated from a previous study (*29*). The starter cultures Viniflora oenos® (O) and Viniflora CH16® (C) are from CHR Hansen (Hørsholm, Denmark), and Lalvin VP41® (V) and Enoferm alpha® (A) are from Lallemand (Stellenbosch, South Africa). The starter cultures were kindly donated by Lallemand and CHR Hansen. In the control wines MLF was prevented through the addition to lyzozyme (0.25 g/L) to the juice to inhibit LAB growth.

6.2.2 Analysis of low-boiling sulfur compounds

The analysis of low-boiling sulfur compounds was described (*31*) and modified (*33*) previously. All analyses were performed in duplicate.

6.2.2.1 Sample preparation

Wine (5 mL, pre-cooled to 4 °C) was transferred into a 10 mL headspace vial containing 1.7 g NaCl and pre-filled with argon 5.0. Ten microliters (10 µL) of a 4 g/L butyl hydroxytoluene (BHT) solution (in ethanol), 10 µL propanal and 10 µL of a satured ethylenediaminetetraacetic acid (EDTA) solution were added as antioxidant, to bind sulfur dioxide and for the complexation of heavy metals, respectively. Lastly, 10 µL of internal standard solution containing 6 µg/L isopropyl methyl sulfide and 6 µg/L butyl methyl sulfide (all in ethanol) was added. To obtain chromatograms containing all sulfur compounds wines were spiked with calibration solutions of low- and high boiling sulfur compounds, respectively. The wines were spiked with the following concentrations: hydrogen sulfide 5.6 µg/L, sulfur dioxide, methane thiole 6.2 µg/L, ethane thiole 10.0 µg/L,: dimethyl sulfide 7.0 µg/L, carbon disulfide 2.0 µg/L, dimethyl disulfide 16.7 µg/L, diethyl disulfide 2.5 µg/L and

20-140 µg/L of all higher boiling sulfur compounds except for methional and methionol which were spiked with 212 µg/L and 2062 µg/L, respectively.

6.2.2.2 GC conditions

An Agilent 6890 (Agilent Technologies, Palo Alto, CA) gas chromatograph equipped with a MPS 2 autosampler for headspace injection and a programmed temperature vaporizing (PTV) injector (CIS 4) both from Gerstel (Mülheim, Germany) was used. Headspace injection of 1000 µL was carried out after conditioning of the sample at 60℃ for 45 min. The GC inlet (CIS4) was operated in solvent vent mode with a split ratio of 10:1. The CIS4 was programmed as follows: initial temperature -100℃, ramped at 12℃/s to 40℃, kept for 1 min, then at 12℃/s to 180℃ held for 8 min. Separation was carried out on a 30 m SPB-1 Sulfur column (Supelco, Belefonte, PA) with an internal diameter (i.d.) of 0.32 mm and a film thickness of 4 µm. Helium was used as carrier gas at a linear gas velocity of 21 cm/s at 60℃. GC oven temperature was programmed as follows: initial temperature 30℃, kept for 7 min, ramped at 10℃/min to 180℃, and held for 10.5 min. The PFPD was operated at 250℃ with 420 kPa air and 420 kPa hydrogen pressures.

6.2.3 Simultaneous analysis of nitrogen and sulfur compounds

The simultaneous analysis of nitrogen and sulfur containing compounds was carried out according to Rauhut co-workers (*32*). Single analyses were performed.

6.2.3.1 Sample preparation

For solid supported liquid-liquid-extraction (SLE) 20 mL wine was spiked with three internal standards and transferred to ChemElut cartridges (20 mL Varian). 4-propylphenol (7 µg/L) was used as internal standard for the nitrogen-compounds and *sec.* butylthiazole (20 µg/L) and 2-methylbenzothiazole (60 µg/L) were used for the sulfur-compounds. After 10 min the analytes were eluted from the cartridge using 20 mL pentane/dichloromethane (2:1). The eluent was concentrated to ~ 1 mL on a Vigereux column and then to about 50 µL in small volume conical flasks in a water bath at 40 ℃ (*34*).

6.2.3.2 GC conditions

Analyses were performed on an Agilent 7890A gas chromatograph equipped with a programmed temperature vaporizing injector (CIS4) from Gerstel. Separation was carried out

on a DB-WAX (J&W Scientific, Folsom, CA) column, 60 m length, 0.32 mm i.d., 0.25 µm film thickness using a temperature gradient with an initial temperature of 60℃ for 1 min, ramped at 3℃/min to 240℃ and kept for 20 min. Helium was used as carrier gas at a constant velocity of 30 cm/s at 60℃. Injection of 2 µL was performed in the "solvent vent" (vent time 10 s) at an injector temperature of 30℃, held for 10 s, after which the temperature of the injector was increased by 12℃/s to 240℃ and held for 300 s (splitless time of 1.5 min). The GC flow was split (1:1) at the end of the GC column to a SCD 350 B chemiluminescence detector (Sievers Research Co., Boulder, CO) for the detection of sulfur-compounds, and an ion trap mass spectrometric detector (GCQ, Thermo Fischer Scientific, San Jose, CA) operated in MS-MS mode for the determination of nitrogen-compounds. MS conditions and parameters were set as follows: EI mode at 70 eV, transfer line 240℃, source temperature: 175℃, emission current: 250 microamps, multiplier 1175 volts, in MS/MS (SIM-SIM) mode, multiplier off-set +300 volts, width ±1 u for all precursor ions. The precursor and product ions used are listed in Table 1.

Table 1: MS-MS parameters for the analysis of nitrogen-containing compounds

Compounds	M_1 -precursor ion m/z	M_2 -product ion m/z	CID[1]
4-propylphenol	136	107	0.90
2AAP	135	120	1.10
methyl anthranilate	151	119	1.10
indole	117	90	0.90
skatole	131	130	0.90

[1] CID: collision induced dissociation, 2AAP: 2-aminoacetophenone

6.2.4 Statistical analysis

Analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test were carried out using the open source software R (version 12.2.1) to determine significant differences in sample means based on the 95% confidence level. For multivariate analysis the FactoMineR package of the open source software R (version 12.2.1) was used. Concentrations of analytes were mean-centred and auto-scaled prior to construction of principal component analysis (PCA) plots in R.

6.3 Result and discussion

Two previous studies focused on the impact of MLF on the volatile composition of the same set of experimental Pinotage wines (*29, 30, 35*). These studies, however, did not include the analysis of sulfur containing compounds. Relatively little known is about the impact of MLF on the concentrations of volatile sulfur compounds. Therefore the same experimental wines produced under controlled conditions with four different MLF starter cultures were analyzed in the present study.

6.3.1 Quantitative analysis of sulfur and nitrogen containing compounds

The two GC methods used in this work allowed the quantification of 20 sulfur compounds and 4 nitrogen compounds. However, only 2 low- and 7 high-boiling sulfur compounds and 3 nitrogen containing compounds were quantified in these wines (Table 2). Figure 1 shows a typical chromatogram obtained for the analysis of low-boiling sulfur compounds in Pinotage wine spiked with various sulfur compounds.

In terms of the compounds detected dimethlysulfide (DMS) can contribute both positively and negatively to wine aroma. Segurel and co-workers (*36*) showed that DMS contributes to the aroma of some red grape cultivars by enhancing “fruity”, “truffle” and “black olive” notes (DMS is also a key aroma compound in truffle (*37*)). However, high concentrations of DMS affect wine aroma negatively (*38, 39*). DMS is produced during fermentation (*40*), but is from produced from precursors such as S-methyl methionine (SMM) during wine aging and storage (*41-43*). San-Juan and co-worker (*44*) showed that DMS in combination with 1-hexanol and methanethiol could be related to the vegetative aroma character of a set of Spanish red wines.

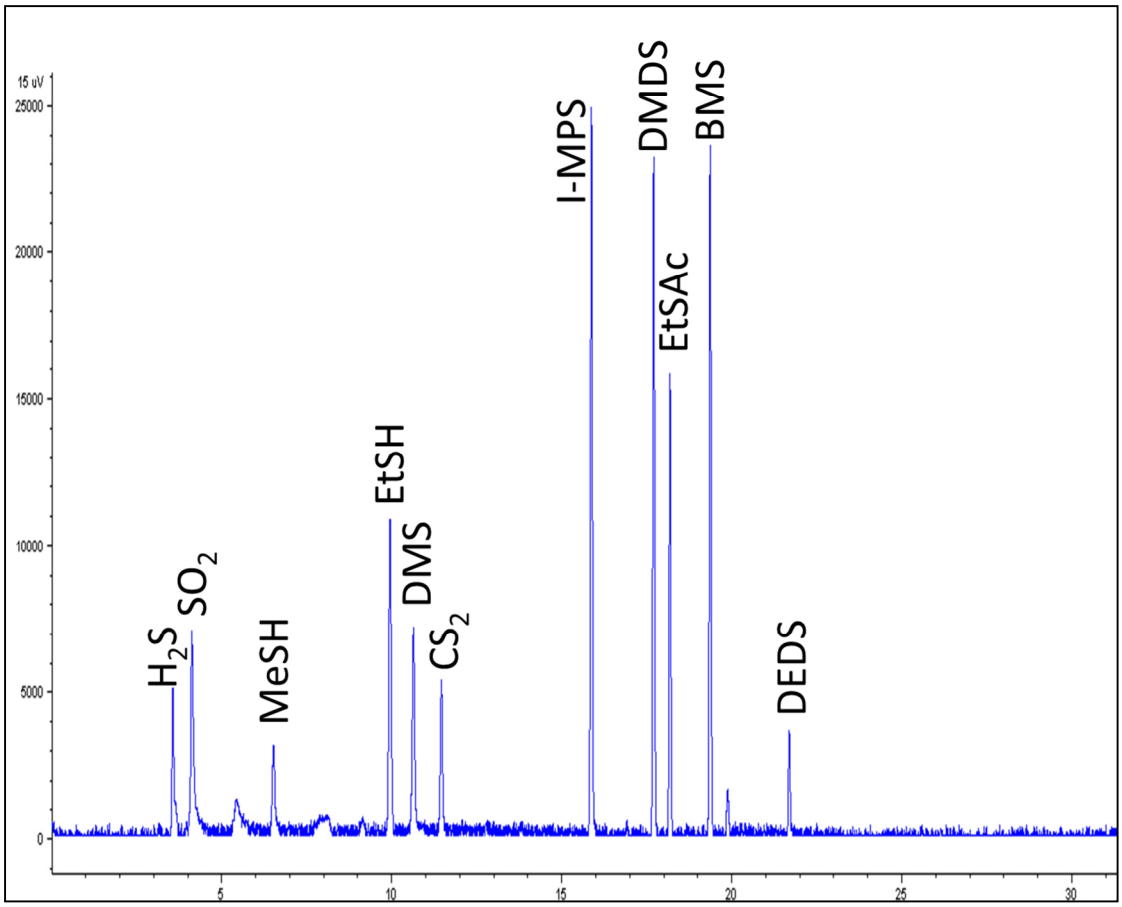

Figure 1: Chromatogram of low boiling sulfur compounds in spiked wine analyzed by HS-GC-PFPD. H_2S: hydrogen sulfide (5.6 µg/L), SO_2: sulfur dioxide, MeSH: methane thiole (6.2 µg/L), EtSH: ethane thiole (10.0 µg/L), DMS: dimethyl sulfide (7.0 µg/L), CS_2: carbon disulfide (2.0 µg/L), I-MPS: isopropyl methyl sulfide (internal standard), DMDS: dimethyl disulfide (16.7 µg/L), BMS: butyl methyl sulfide (internal standard), DEDS: diethyl disulfide (2.5 µg/L)

Chromatograms of the simultaneous analysis of high-boiling sulfur- and nitrogen compounds are presented in Figure 2 and Figure 3, respectively. The quantified compounds are discussed below.

Thioesters can contribute to increasing or re-occurring off-flavors during storage after treatment and bottling. This is due to the equilibrium-dependent hydrolysis of, for instance, thioacetic acid esters to produce thiols and acetic acid. Thiols have lower odor thresholds (> 2 µg/L) than thioacetic acid esters (> 40 µg/L), therefore the release of only small amounts of thiols are sufficient to provoke sulfur off-flavors (*45*).

Additionally, cyclic and heterocyclic sulfur compounds have also been linked to objectionable wine aroma. Benzothiazole (BTH) occurs in many foodstuffs. Its odor descriptors are “rubbery” and reminiscent of quinolone. The source of BHT in wine is not clear, although it may be formed for instance by non-enzymatic browning reactions, thermal reaction of

cysteine with dicarbonyl compounds or thermal degradation of thiamine (*46*). The production of this thiazole derivative by several microorganisms has also been reported. (*47*, *48*).

Dihydro-2-methyl(2H)thiophen-3-one is described by odor descriptors of “chlorine”, “wet”, “ozone” (*49*), “sour-fruity”, “musty”, “green” (*50*) and “sulfur”, and also “fruity” and “berry” (*51*). In orange juice it was identified as a degradation product of thiamine (*50*). In addition to other S-compounds, it was detected in fermentation experiments with methionine as the only nitrogen source (*52*). Li and co-workers (*51*) reported the formation of this compound during the fermentation of mango juice with different *Sacharomyces cerevisiae* strains, as they did not detect it in the unfermented juice.

Cis/trans tetrahydro-2-methylthiophene-3-ol was reported to be present in higher concentrations in wines with sulfide off-flavors (*23*, *45*). Odor descriptors such as “cheese”, “sweaty” and “negatively” reminiscent of “leeks” for the *cis* isomer, and “sweet”, positively reminiscent of “leeks” and “spices” for the *trans* isomer were reported (*53*). The isomers are produced in a 2:1 ratio (*cis*:*trans*) during fermentation (*54*) in (*55*).

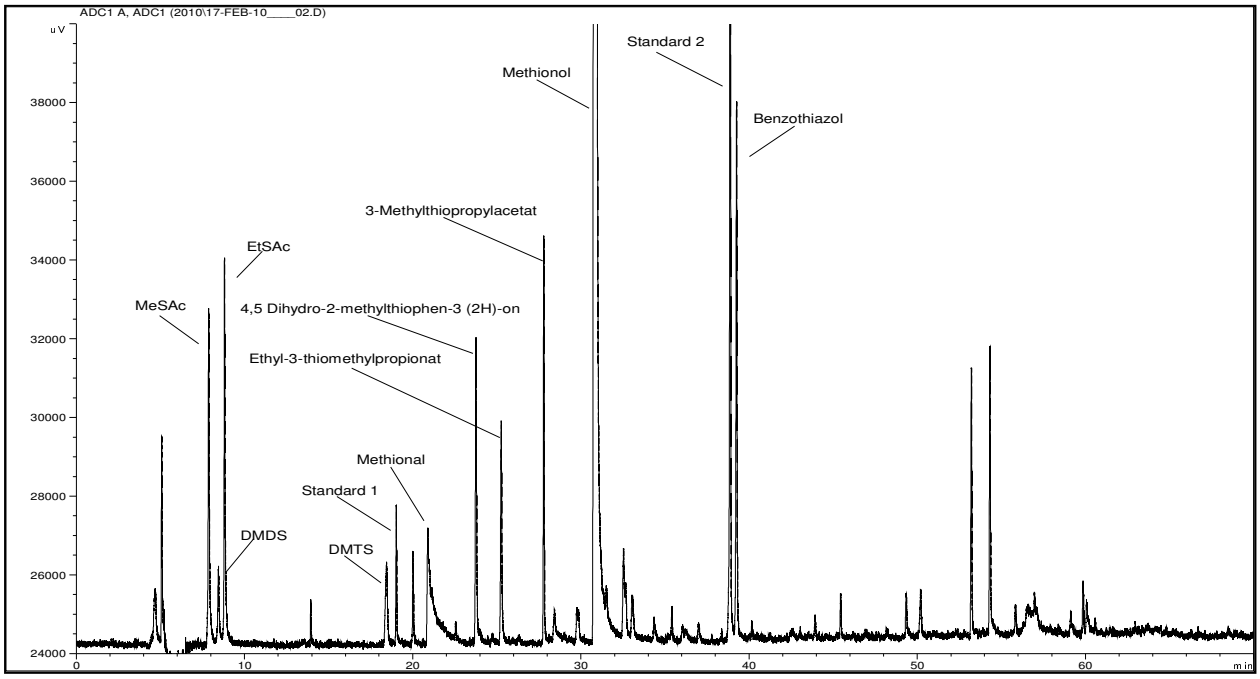

Figure 2: Chromatogram of high boiling sulfur compounds in wine (spiked with 20-140 µg/L of all compounds except for methional and methionol which were spiked with 212 µg/L and 2062 µg/L, respectively) analyzed by SLE-GC-SCD. MeSAc: methyl thioacetate, EtSAc: ethyl thioacetate, DMDS: dimethyl disulfide, DMTS: dimethyl trisulfide, Standard 1: 2-sec buthylthiazole, Standard 2: 2-methylbenzothiazole.

The nitrogen containing compound 2-aminoacetophenone (2AAP) is a key compound associated with the atypical aging off-flavor in wine, and is often described as contributing

"acacia blossom", "naphthalene-like", and "furniture polish" odor. Many factors influence the occurrence of higher 2AAP concentrations in wine, such as reduced nitrogen fertilization, drought stress, hot conditions and early harvest. It can be formed in wine during and after alcoholic fermentation, where the phytohormone indole-3-acetic acid (IAA) plays a role as precursor (*56*, *57*). Rapp demonstrated the formation of small amounts of 2AAP by *Saccharomyces cerevisiae* in model solutions containing only tryphtophan as nitrogen source (*58*). It is, however, assumed that due to different attributes described for the atypical aging off-flavors, other nitrogen compounds such as indole, skatole, and anthranilic acid esters may also be involved. Methylanthranilate (together with 2AAP) is related to the foxy-taint of American hybrids, but has also been detected in wines from *Vitis vinifera* (*59*, *60*). Pure 2AAP has also been described as contributing grapelike odor (*61*).

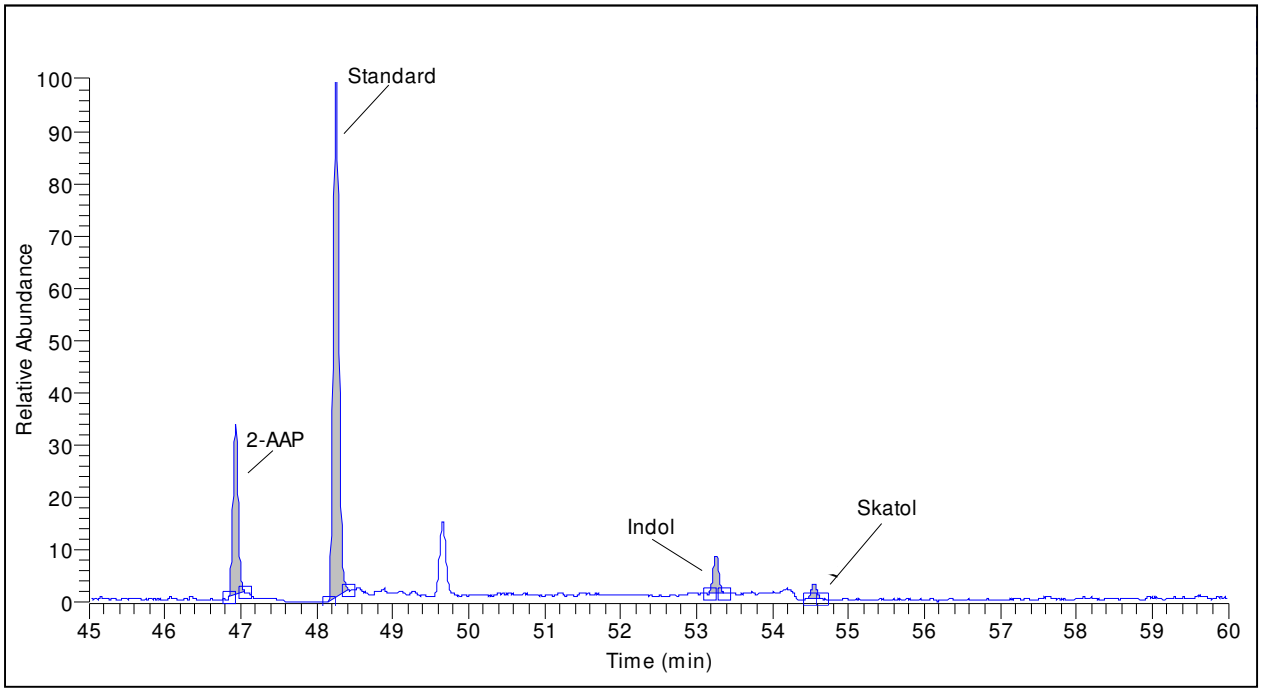

Figure 3: Chromatogram of nitrogen compounds in wine analyzed by SLE-GC-MS/MS. 2-AAP: 2-aminoacetophenone, Standard: 4-propylphenol.

Table 2. List of compounds quantified in Pinotage wine samples by two different GC methods. Alphabetic letters row wise indicate significant differences ($p<0.05$) in the sample means for triplicate biological repeats. Values are represented as mean levels in µg/L ± standard deviation.

No.	Compound		Ctrl Average ±SD	C Average ±SD	V Average ±SD	A Average ±SD	O Average ±SD
	sulfur containing compounds						
1	hydrogen sulfide	1			not detected		
2	methanethiol	1			not detected		
3	ethanethiol	1			not detected		
4	dimethyl sulfide [DMS]	1	8.23 ±0.06 c	8.87 ±0.15 b	9.07 ±0.23 ab	8.97 ±0.12 b	9.33 ±0.15 a
5	cabon disulfide	1	1.93 ±0.15 n.s.	3.00 ±2.60 n.s.	1.57 ±0.42 n.s.	1.73 ±0.42 n.s.	1.73 ±0.23 n.s.
6	Methyl thioacetate	1			not detected		
7	dimethyl disulfide [DMDS]	1			not detected		
8	Ethyl thioacetate	1			not detected		
9	dimethyl disulfide [DEDS]	1			not detected		
10	dimethyl trisulfide [DMTS]	1			not detected		
11	Propanal, 3-(methylthio)- [methional]	2			not detected		
12	dihydro-2-methyl-3(2H)-thiophenone	2,3	0.52 ±0.07 c	0.82 ±0.08 ab	0.69 ±0.04 abc	0.62 ±0.11 bc	0.83 ±0.20 a
13	methyl 3-(methylthio)propionate	2	8.30 ±1.95 c	25.27 ±3.76 a	20.87 ±1.70 ab	20.87 ±2.54 ab	19.93 ±3.35 bc
14	ethyl 3-(methylthio)propionate	2	2.60 ±0.26 n.s.	2.77 ±0.59 n.s.	2.40 ±0.10 n.s.	2.30 ±0.36 n.s.	2.03 ±0.06 n.s.
15	3-(methylthio)propyl acetate	2			not detected		
16	3-(methylthio)propanol [methionol]	2	1245.0 ±64.8 c	2819.7 ±437.8 ab	3335.3 ±430.6 a	2522.3 ±189.7 b	2433.3 ±67.5 b
17	(Z)-tetrahydro-2-methyl-thiophen-3-ol	2,3	0.09 ±0.01 c	0.16 ±0.01 a	0.16 ±0.02 a	0.13 ±0.01 b	0.15 ±0.02 ab
18	3-(ethylthio)-1-propanol [ethionol]	2			not detected		
19	(E)-tetrahydro-2-methyl-thiophen-3-ol	2,3	0.07 ±0.00 b	0.12 ±0.01 a	0.12 ±0.00 a	0.12 ±0.02 a	0.12 ±0.01 a
20	benzothiazole	2	1.37 ±0.42 b	2.27 ±0.72 ab	3.30 ±0.89 a	3.10 ±0.20 a	1.83 ±0.58 b
	nitrogen containing compounds						
21	2-aminoacetophenone [2-AAP]	2	0.17 ±0.01 c	0.39 ±0.10 b	0.09 ±0.02 c	0.44 ±0.06 b	0.59 ±0.02 a
22	Anthranilic acid methylester	2			not detected		
23	indole	2	0.14 ±0.01 b	0.25 ±0.05 a	0.23 ±0.06 a	0.21 ±0.01 ab	0.21 ±0.01 a
24	skatole	2,3	0.02 ±0.01 n.s.	0.03 ±0.01 n.s.	0.03 ±0.01 n.s.	0.03 ±0.01 n.s.	0.03 ±0.00 n.s.

[1]GC method for low-boiling S-compouns used for quantification. [2]GC methods for high-boiling S-compounds used for quantification. [3]Values are presented as peak area ratios of the compounds calculated relative to the internal standard. n.s. not significant.

Regarding the interpretation of the data presented in Table 2, it must be noted that the wines were bottled 18 months prior to analysis. During wine aging volatile sulfur constituents can be involved in several chemical reactions. For example, thioesters can hydrolyze to produce free thiols at low pH during bottle aging (*55, 62*). This aspect should be cautiously considered when comparing the quantitative data reported here with literature data. Nonetheless, during this study all analyses were performed within two weeks, ensuring consistency within the dataset and allowing accurate comparison between the investigated wines.

6.3.2 Statistical analysis of quantitative data

Statistical analyses were carried out by means of analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test and principle component analysis (PCA). Analysis of variance showed significant differences in the mean levels of 9 out of 12 quantified compounds between the different treatments (the control and the four wines fermented with different MLF starter cultures). PCA led to grouping between MLF samples and the control as well as to grouping of the MLF samples themselves.

6.3.2.1 Principal component analysis

Principal component analysis using only variables differing significantly between the samples allowed not only differentiation between the control (no MLF) and the MLF wines, but also between the MLF wines produced with different starter cultures (Figure 4 and Figure 5). Note that categorical supplementary variables for the individual wine samples (supplementary individuals) were added to the dataset. These supplementary individuals were used to categorize the biological repeats of each wines produced with different starter cultures. Supplementary information does not intervene in any way with the PCA model, but is merely used to simplify interpretation of PCA results. Additionally, calculated squared cosine values were used to evaluate the importance of a principal component for the variance of an individual (wine sample) or variable (compound) (*63*). Higher squared cosine values indicate a more significant link with the corresponding principal component. The squared cosines of supplementary individuals (wines) and variables (compounds) on PC 1, PC 2 and PC 3 are represented in Table 3 (high values are indicated in bold type).

Table 3. Squared cosines for variables (compounds) and supplementary individuals (wines) on PC 1 to PC 3 (high values are printed in bold type). For convenience all values have been multiplied by 100 and rounded.

No.	Variables (Compound)	squared cosine[1]			Individuals[2] (Wines)	squared cosine[1]		
		PC 1	PC 2	PC 3		PC 1	PC 2	PC 3
4	dimethyl sulfide (DMS)	**73**	3	4	A	11	7	**60**
12	dihydro-2-methyl-3(2H)-thiophenone	**48**	**24**	9	C	**67**	3	8
13	methyl 3-(methylthio)propionate	**85**	1	1	Ctr	**100**	0	0
16	3-(methylthio)propanol [methionol]	**79**	**11**	5	O	31	**60**	3
17	(Z)-tetrahydro-2-methyl-thiophen-3-ol	**85**	0	**10**	V	33	**56**	7
19	(E)-tetrahydro-2-methyl- thiophen-3-ol	**91**	1	1				
20	benzothiazole	**29**	**52**	**12**				
21	2-aminoacetophenone (2-AAP)	17	**58**	**22**				
23	indole	**48**	5	3				

[1] multiplied by 100 and rounded; [2] supplemtary individuals.

Figure 4a provides a plot of individuals (wines) for the first two PCs and Figure 4b shows variables (compounds) on a correlation circle (radius = 1), where the length of an arrow signifies the degree of correlation. Both plots essentially constitute the traditional biplot, but are presented separately to prevent cluttering of the plot. Efficient grouping was obtained for all biological repeats of the MLF wines fermented with starter cultures V, O and C as well as for the control wines. Note that clustering of the MLF wines fermented with starter culture A in the center of the graph indicates insufficient explanation of their variance by the first two components. However, PC 1 completely separates the control from the MLF wines. The squared cosine value in Table 3 show that the first component accounts for the complete differentiation between the control and MLF wines. Examination of the variables plot shows clear positive correlation of the compounds DMS (compound **4**), methyl 3-(methylthio) propionate (**13**), (Z)-tetrahydro-2-methyl-thiophen-3-ol (**17**), (E)-tetrahydro-2-methyl-thiophen-3-ol (**19**), indole (**23**), methionol (**16**) and dihydro-2-methyl-3(2H)-thiophenone (**12**) with PC 1. Higher concentrations of these compounds were measured in all MLF wines. The squared cosine values show that PC 1 also contributes partially to the differentiation of the MLF wines produced with starter culture C. PC 2 explains mainly the variance in quantitative data between wines fermented with starter cultures O and V. This differentiation is primarily due to differences in the levels of 2AAP (**21**) and benzothiazole (**20**) between these wines (the former is lower and latter higher in wines produced from starter culture V).

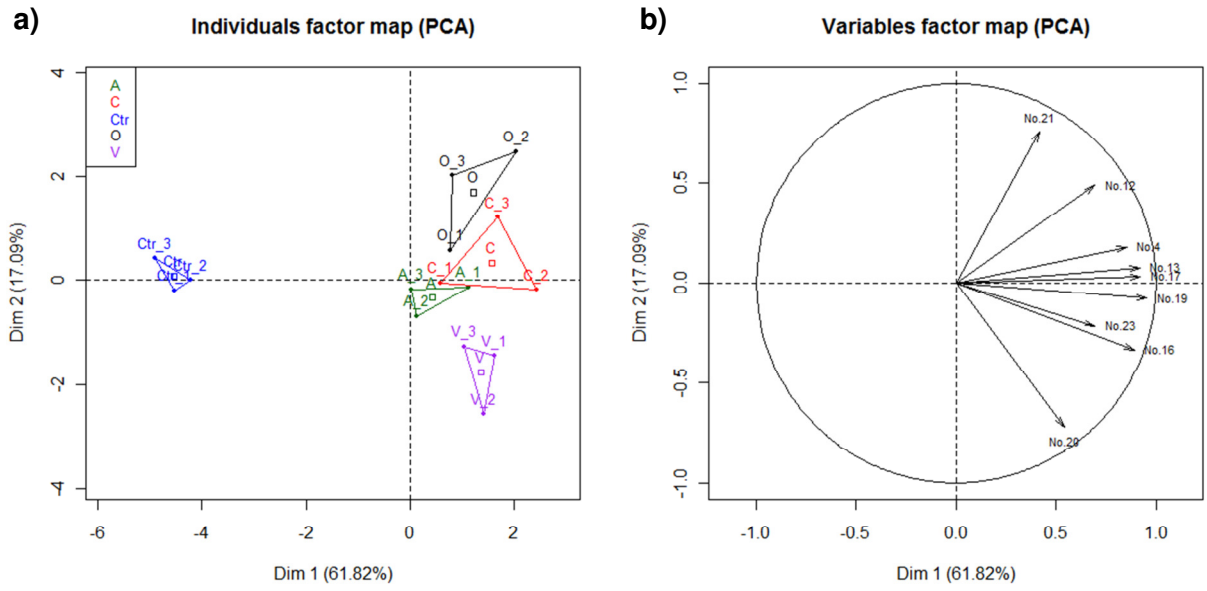

Figure 4: PCA results. (a) Individuals (□: supplementary individuals) and (b) variables (compounds) on a correlation circle (radius = 1) plotted on PC 1 and PC 2, for the data of sulfur and nitrogen compounds. Starter cultures: Viniflora oenos® (O) and Viniflora CH16® (C), Lalvin VP41® (V) and Enoferm alpha® (A), control = no MLF (Ctr)

Figure 5 shows the individual and variable plots for PC 2 and PC 3, which provide more clarity regarding the differences between the MLF wines. The control wine and MLF wines fermented with starter culture C are located in the center of the graph, which denotes that their variances are not explained by these two PCs. Hence PC 2 contributes to differentiation of MLF wines produced with starter cultures O and V, and PC 3 contributes to differentiate the MLF wines produced with starter culture A, which is also indicated by the squared cosine values (Table 3). The wines fermented with starter cultures V and O are best differentiated according to PC 2. A closer look at the variable plot shows that the position of wines fermented with starter culture V are a result of negative correlation with 2AAP (**21**) and dihydro-2-methyl-3(2H)-thiophenone (**12**) and positive correlation with methionol (**16**) and benzothiazole (**20**). On the other hand, the wines from starter culture O correlate positively with 2AAP (**21**) and dihydro-2-methyl-3(2H)-thiophenone (**12**) and negatively with methionol (**16**) and benzothiazole (**20**). The MLF wines from starter culture A, for which differentiation mainly occurs due to PC 3, correlate negatively with (Z)-tetrahydro-2-methyl-thiophen-3-ol (**17**), and positively with benzothiazole (**20**) and 2AAP (**21**).

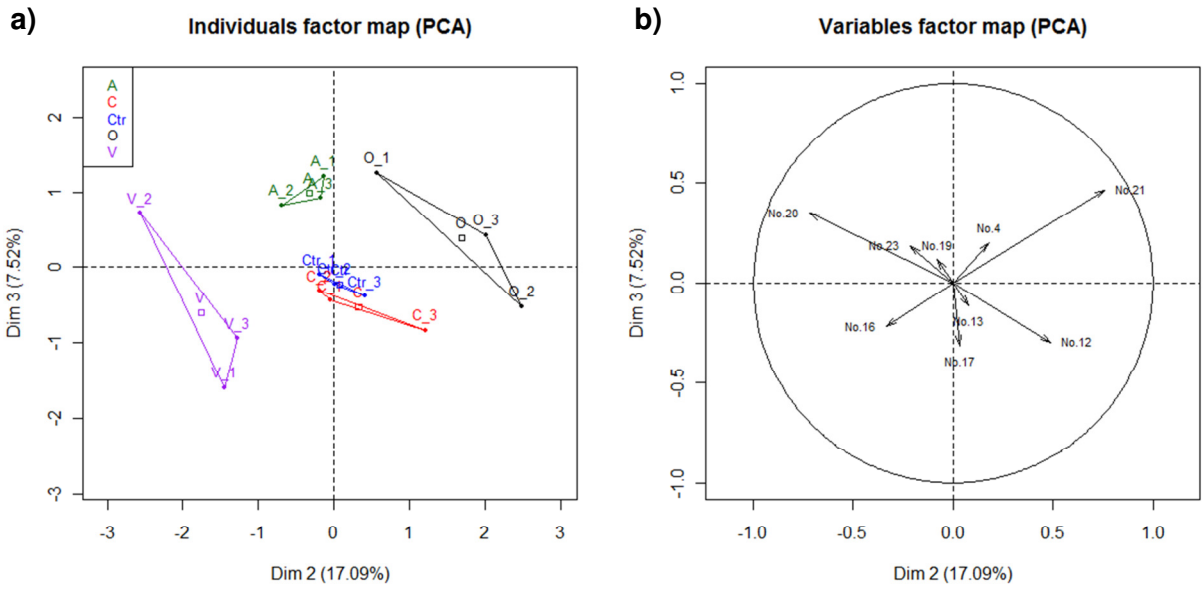

Figure 5: PCA results. (a) Individuals (□: supplementary individuals) and (b) variables (compounds) on a correlation circle (radius = 1) plotted on PC 3 and PC 3, for the data of sulfur and nitrogen compounds. Starter cultures: Viniflora oenos® (O) and Viniflora CH16® (C), Lalvin VP41® (V) and Enoferm alpha® (A), control = no MLF (Ctr)

6.3.2.2 ANOVA *post hoc* comparison: Fisher's least significant difference

Following one-way analysis of variance (ANOVA), means of the content values for the compounds were also compared between the different wines using the Fisher's least significant difference (LSD) test (Table 2). Analogous the results obtained by PCA, the following compounds were present at significantly higher concentrations in all MLF wines compared to the control: DMS (**4**), methyl 3-(methylthio)propionate (**13**), (Z)-tetrahydro-2-methyl-thiophen-3-ol(**17**), (E)-tetrahydro-2-methyl-thiophen-3-ol(**19**), indole (**23**), methionol (**16**) and dihydro-2-methyl-3(2H)-thiophenone (**12**).

The concentration of two of these compounds also differed significantly between the MLF samples. Methionol (**16**) showed the highest concentrations in wines fermented with starter culture V and concentrations of (Z)-tetrahydro-2-methyl-thiophen-3-ol(**17**) were lower in the MLF wines of starter culture A. Essentially the differences between the MLF wines are represented by these two compounds, benzothiazole (**20**), 2AAP (**21**) and dihydro-2-methyl-3(2H)-thiophenone (**12**). Benzothiazole (**20**) concentrations were the highest in the wines produced with starter cultures V and A. 2AAP (**21**) showing the highest concentrations in wines fermented with starter culture O, followed by equal amounts for wines A and C. The concentrations of this compound in the control and the MLF wines V were not significantly

different. Dihydro-2-methyl-3(2H)-thiophenone (**12**) concentrations were significantly higher in the wines produced from starter cultures O and C.

Considering the absolute increase of concentration of the different compounds only methionol (**16**), methyl 3-(methylthio)propionate (**13**) and 2AAP (**21**) showed relatively large concentration differences between the wines. The absolute increase in concentrations of all other compounds was very low, albeit significant.

The concentrations of both methionol (**16**) and methyl 3-(methylthio)propionate (**13**) increased 2-3 times following MLF in all wines, resulting in concentrations of 2500-3000 µg/L and 20-25 µg/L, respectively. It has been shown that *O. oeni* is able to metabolize methionine (*64*) to form methionol and 3-(methylthio)propionic acid (*13*). Methyl 3-(methylthio)propionate (**13**) is the corresponding ester of 3-(methylthio)propionic acid, and is therefore most likely formed from this acid, either by enzymatic or chemical esterification. Interestingly, the concentration of ethyl 3-(methylthio)propionate (**14**) showed no significant differences between the different treatments and 3-(methylthio)propyl acetate (**15**) was not detected in the wines.

The concentration of 2AAP (**21**) was 0.4-0.6 µg/L in the MLF wines fermented with starter cultures C, A and O, but only 0.1-0.2 µg/L in the control wine and the MLF wines produced with starter culture V. The impact of MLF on the concentration of 2AAP has not been reported previously. The reason for these differences due to MLF therefore still needs to be investigated. Interestingly, starter culture V was previously reported to have a different impact on the volatile composition of the same wines compared to the other starter cultures used here (*30*), which could indicate metabolic diffrences of this starter culture compared to the others used in this study. 2AAP is linked to the “atypical aging” off-flavor and has an odor threshold of 0.5-1.5 µg/L in wine (*65*). This compound is known to be formed mainly of the grape derived plant hormone indole-3-acetic acid (IAA), but other, less important, formation pathways are also known (*57*). The oxidative degradation of IAA to 2AAP is caused by superoxide radicals, which are formed in wine by co-oxidation of sulfite to sulfate following the addition of sulfur dioxide. In red wine these superoxide radicals are scavenged by polyphenolic compounds (*57*), therefore 2AAP levels in red wine are usually much lower than in white wines. It was shown that an addition of substances with antioxidative activity such as ascorbic acid to wine inhibits the formation of 2AAP (*56, 66*). The microbial formation of 2AAP out of the amino acid tryptophan by yeast (*58*) (in wine) and bacteria such as the pathogenic bacterium *Pseudomonas aeruginosa* (*61*) (in culture and in burn wounds) has also been reported. Schmarr and co-workers (*67*) described an impact of the pH of wine during sample preparation for GC analysis of 2AAP on the detected concentration. However, it is unlikely that in this study the pH of the wines are responsible for the observed changes, as pH’s between the control wines and all MLF wines differed only by ~ 0.1 pH units (*29*).

Therefore no conclusions regarding the reason for the change of the concentration of 2AAP following MLF could be made at this stage.

Pripis-Nicolau and co-workers (*13*) reported trace amounts of DMS (**4**) and other sulfur containing compounds in a basal medium inoculated with LAB strains, but they did not correlate their formation with the methionine metabolism of LAB. A chemical pathway can also be involved in the formation of DMS (**4**) and other sulfur compounds (*68*). Therefore it is unclear whether the sulfur compounds which increased only to minimal extents following MLF in this study are formed biotically or abiotically

6.4 Summary and conclusions

The goal of this study was to investigate the effect of MLF on the composition of volatile sulfur and selected nitrogen compounds in wine. Previous studies on these wines used targeted GC analysis for major volatiles and carbonyl compounds, as well as untargeted comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS) analysis. However, these methods failed to provide the requisite selectivity for the analysis of volatile sulfur and nitrogen compounds. For this purpose, selective methods (detectors) were used.

Both GC methods, one using headspace injection and PFPD detection for accurate quantification of low-boiling sulfur compounds, and a second for simultaneous analysis of high-boiling S- and N-compounds using SLE together with sulfur chemiluminescence and MS/MS detection, proved to be suitable to quantify important odor compounds in wine in previous studies. Headspace injection for low-boiling sulfur compounds in combination with a temperature programmed GC inlet minimized discrimination during sample introduction. For the simultaneous analysis of high-boiling sulfur and nitrogen containing compounds, SLE provided excellent extraction efficiency and reduced liquid handling issues associated with traditional liquid-liquid extraction (LLE). Chemiluminescence and pulsed flame photometric detection were beneficial detectors due to their selective detection of sulfur containing compounds, respectively. Both methods have been successfully applied to study the impact of different MLF starter cultures on volatile hetero-atomic compounds in Pinotage wines.

This study outlines for the first time changes of some important sulfur and nitrogen containing compounds following MLF in Pinotage wines. Results of ANOVA followed by LSD testing and PCA showed significant differences in the composition volatile sulfur-containing compounds. Nine sulfur and 3 nitrogen compounds were quantified, levels of 7 sulfur and 2 nitrogen compounds were significantly higher in the MLF wines. The amounts of methionol (**16**),

methyl 3-(methylthio)propionate (**13**) and 2AAP (**21**) increased considerably following MLF, whereas the quantitative increases of all the other compounds were very low.

Increased concentrations of methionol (**16**) in the MLF wines are in agreement with published results on the methionine metabolism of LAB. It is especially noteworthy that the concentration of methyl 3-(methylthio)propionate (**13**) and methionol (**16**) increased by the factor of 2-3 following MLF. Methyl 3-(methylthio)propionate (**13**) has not previously been linked to MLF. Furthermore, levels of 2AAP increased 4-5 times in three of the four wines fermented with different MLF starter cultures compared to the control wines. Only the wines obtained from starter culture V did not show significantly different levels of 2AAP compared to the control, which could indicate significant metabolic differences of this strain compared to the other strains. In a previous study involving the same wines, starter culture V was also reported to have a different impact on the volatile composition of wine, which could indicate metabolic diffrences of this starter culture compared to the others used in this study. Note that 2AAP plays an important role as a key compound in wines with an “atypical aging” off flavor. Changing levels of 2AAP following MLF are also reported here for the first time.

6.5 References

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7

General conclusions

The primary goal of this study was the evaluation of the potential of comprehensive two-dimensional gas chromatography coupled to time-of-flight spectrometry (GC×GC-TOF-MS) for the analysis of wine volatiles. GC×GC-TOF-MS was used to investigate the impact of malolactic fermentation (MLF) on the volatile composition of Pinotage wines and the evaluation of a new stir bar sorptive extraction (SBSE) phase for the analysis of wine volatiles.

In the first few chapters general background on the principles of gas chromatography with emphasis on multidimensional methods and sample preparation is provided (chapter 2). Furthermore, the volatile composition of wine and the current knowledge of the impact of malolactic fermentation on volatile wine constituents are reviewed in chapter 3.

The main part of the research is presented in chapter 4. This chapter contains the profiling of volatile compounds in MLF fermented wines. The impact of MLF on the volatile composition of wine is not well understood and very little information is available regarding the influence of MLF on Pinotage wines in particular. In this study a set of Pinotage wines fermented with several commercial LAB starter cultures under controlled conditions was therefore used. The utilization of solid phase microextraction in combination with comprehensive two-dimensional gas chromatography coupled to time-of-flight spectrometry (HS-SPME-GC×GC-TOF-MS) provided a powerful tool for the analysis of these wines. The immense separation power obtained by orthogonal separations in GC×GC allowed the identification of 115 compounds. Moreover, enhanced sensitivity obtained by GC×GC separation allowed the analysis of compounds present in low concentrations. The identified compounds include esters, alcohols, carbonyl compounds, acids, furanes, nitrogen-containing compounds and terpenoids. The use of GC×GC led to the identification of a much larger number of compounds compared to a one-dimensional gas chromatography and the techniques therefore clearly shows promise for the detailed investigation of wine volatiles.

Furthermore, 60 compounds were quantified relative to an internal standard. The accuracy of automated peak integration was found to be insufficient for accurate statistical analysis and manual intervention was required. Subsequent combination of quantitative data with univariate and multivariate statistical data analysis allowed the distinction between wines fermented with different starter cultures and identification of the compounds responsible for this differentiation. These results point to significant metabolic differences between the starter cultures. Some compounds which showed significant difference between control and MLF wines fermented with different starter cultures, such as some minor esters, aldehydes and ketones, were linked to malolactic fermentation for the first time.

The evaluation of the suitability of a new phase for stir bar sorptive extraction (SBSE) for the analysis of wine volatiles is presented in chapter 5. The recently introduced dual-phase polar

EG-Silicone Twister® was compared with the conventional polydimethylsiloxane (PDMS) Twister. To achieve detection of a wide range of volatile wine constituents GC×GC-TOF-MS with thermal desorption (TD) was used. Several instrumental problems were encountered in the combination of the SBSE and GC×GC, although a large number of compounds were tentatively identified. The comparison of absolute peak areas demonstrated the higher affinity of especially more polar compounds for the EG-Silicone phase. However, the thermal instability resulting in interfering degradation products hampers the usage of this phase together with mass spectrometry or other non-selective detectors.

Based on these results, the suitability of the new twister phase for the extraction of thiazole, 4-methylthiazole and 2,4-dimethylthiazole from wine was further evaluated (chapter 5). Selective nitrogen-chemiluminescence detection (NCD) with two-dimensional heart-cutting gas chromatography was successfully used to overcome the drawbacks associated with interfering degradation products of the EG-Silicone phase. The extraction performance of the EG-Silicone Twister was compared to conventional PDMS Twister in headspace and immersion modes. The EG-Silicone Twister showed much better extraction properties for all three compounds. The most polar compound, thiazole, could not be detected when using the PDMS phase. Despite the advantages of the new stir bar phase for the extraction of the thiazoles, the developed method was not sufficiently sensitive to allow analysis at the natural levels of these compounds in wine.

In the final research chapter (chapter 6), further investigation of the MLF Pinotage wines was performed. Very little is known on the impact of MLF on the composition of volatile sulfur and nitrogen compounds. Since HS-SPME-GC×GC-TOF-MS was not suited for the analysis of these highly specific wine volatiles, two established one-dimensional GC methods using headspace injection and liquid-liquid extraction in combination with sulfur selective detection were used for the analysis of sulfur- and nitrogen-containing compounds. These methods provided quantitative data for a number of important hetero-atomic compounds in the experimental wines. Similar to the HS-SPME-GC×GC-TOF-MS results, univariate and multivariate statistical methods enabled the distinction between different starter cultures based on quantitative data for 12 compounds. These data further strengthened the assumption of significant metabolic differences between the starter cultures. This study reports for the first time changes of sulfur and nitrogen containing compounds in Pinotage wines following MLF. Furthermore, differences in the concentrations of the compounds methyl 3-(methylthio)propionate and 2-aminoacetophenone were linked to MLF for the first time.

Several general conclusions may be drawn from the results presented in this thesis. In the first instance, GC×GC was shown to be a very promising technique for the analysis of wine volatiles, since it allows the separation and identification of a large number of compounds,

including trace-level volatiles, in a single analysis. Despite these advantages, however, accurate quantification of GC×GC-TOF-MS data requires extensive manual intervention, which results in intensive data analysis. There is still a need for software capable of automated non-targeted analysis of GC×GC data for screening purposes. Future combination of GC×GC with selective detectors could open a new door for the analysis of hetero-atomic compounds in wine.

Data reported here for the analysis of Pinotage wines using GC×GC and selective methods for the analysis of sulfur- and nitrogen-containing compounds have contributed significant new information regarding the effect of malolactic fermentation on the volatile compounds in these wines. Future studies in this field should focus on the metabolism of the bacterial starter cultures and the evaluation of their impact on sensory properties of wine.

Finally, the new EG-Silicone Twister phase was found to offer a useful alternative for targeted analysis of more polar compounds such as hetero-atomic compounds in wine, especially in combination with selective detectors such as sulfur and nitrogen-chemiluminescence or flame photometric detectors.