



PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
ESCUELA DE INGENIERÍA

IDENTIFICATION AND CHARACTERIZATION OF ODORANT COMPOUNDS IN CARMENERE RED WINE

CAROLINA PAVEZ MORENO

Thesis submitted to the Office of Research and Graduate Studies in partial
fulfillment of the requirements for the Degree of Doctor in Engineering
Sciences

Advisor:

EDUARDO AGOSIN TRUMPER

Santiago de Chile, December, 2014

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CAROLINA PAVEZ MORENO

Members of the Committee:

EDUARDO AGOSIN TRUMPER

EDMUNDO BORDEU SCHWARZE

FELIPE LAURIE GLEISNER

FRANCO PEDRESCHI PLASENCIA

RICARDO TAPIA APATI

MARTIN STEINHAUS

CRISTIAN VIAL EDWARDS

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences.

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*A Juan y Alejandra, que me enseñaron
que no existen los sueños imposibles...*

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ABBREVIATIONS

AEDA	Aroma Extract Dilution Analysis
ASTM	American Society for Testing and Materials
AV	Acidic Volatiles
CI	Chemical Ionization
CoA	Coenzyme A
DB-5	(5% Phenyl)-methylpolysiloxane
DB-1701	(14% Cyanopropyl-phenyl)-methylpolysiloxane
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DVB/PDMS	Divinylbenzene/Polydimethylsiloxane
ECD	Electron Capture Detector
EDTA	Ethylenediaminetetraacetic
EI	Electron Impact Ionization Mode
eV	Electron Volt
FD	Flavor Dilution
FFAP	Free Fatty Acid Phase
FID	Flame Ionization Detector
g	Gram
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GC-O	Gas Chromatography-Olfactometry
GC-NCI-MS	Gas Chromatography-Negative Chemical Ionization-Mass Spectrometry
HP-1	100% Dimethylpolysiloxane
HRGC-FID	High Resolution Gas Chromatography-Flame Ionization Detector
HRGC-MS	High Resolution Gas Chromatography-Mass Spectrometry
HRGC-O	High Resolution Gas Chromatography-Olfactometry
id	Internal Diameter
ISO	International Organization for Standardization
KFO	Key Food odorants

LOD	Limit of Detection
LOQ	Limit of Quantitation
M	Mol per Liter
MCSS	Moving Column Stream Switching
mg/L	Milligram per Liter
mL	Milliliter
mmol/L	Millimol per Liter
MS	Mass Spectrum
m/z	Mass-to-Charge Ratio
NBV	Neutral/Basic Volatiles
NCI	Negative Chemical Ionization
ng/L	Nanogram per Liter
OAV	Odor Activity Value
OT	Odor Threshold
PCA	Principal Component Analysis
PFBBBr	Pentafluorobenzyl Bromide
R ²	Coefficient of Determination
RI	Retention Index
RSD	Residual Standard Deviation
PTFE	Polytetrafluoroethylene
SAFE	Solvent Assisted Flavor Evaporation
SIDA	Stable Isotope Dilution Assay
SIM	Single Ion Monitoring
S/N	Signal-to-Noise Ratio
SPE	Solid Phase Extraction
SPME	Solid Phase Micro Extraction
μg/L	Microgram per Liter
μL	Microliter
v/v	Volumen/Volumen

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ESCUELA DE INGENIERÍA

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Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of
the requirements for the Degree of Doctor in Engineering Sciences by

CAROLINA PAVEZ MORENO

ABSTRACT

Wine aroma is one of the most important quality attributes that determine the consumer acceptance and the wine's market segment and the final price. Chile ranks fourth in 2013, among the major wine export countries behind France, Italy and Spain. According to the last report of the International Organization of Vine and Wine (OIV), Chilean wine exports were close to US\$ 1,900 million in 2013 with USA, England and China as the major export destinations.

Vitis Vinifera Carmenere is a red wine variety originating from the Bordeaux region of France. Carmenere was thought to be extinct after the phylloxera (*Daktulosphaira vitifoliae*) plague in Europe, but in 1994, it was rediscovered in Chile, where most of the worldwide vineyards of this grape variety are currently planted. The aroma of this cultivar has been described as vegetable-like, spicy and fruity and, altogether, these aroma descriptors characterize this wine variety. Although the odorant compounds related to Carmenere's vegetable-like aromas, as well as their sensory thresholds and concentration values, have been well addressed, there is scarce information about the identity of the volatile compounds related to fruity and spicy descriptors.

The identification of the main odorants related to Carmenere's fruity aromas is critical to manage and improve the quality of this wine variety. Therefore, the aim of this study was to identify and characterize the main odorant zones of Carmenere red wine focusing

on those related with fruity descriptors. For this purpose, we first screened the major odorants present in a varietal Carmenere wine by gas chromatography-olfactometry and aroma extract dilution analysis. Among all odorants identified, special emphasis was placed on the odorants methyl 2-methylbutanoate and methyl 3-methylbutanoate, reported for the first time in red wines, because these two novel ester compounds could act as enhancers of fruity aromas in red wines.

Additionally, previous studies on volatile thiols in red wines have reported that the compound 4-methyl-4-sulfanyl-2-pentanone could be the responsible of blackcurrant aroma, as well as the compounds 3-sulfanyl-1-hexanol and 3-sulfanylhexyl acetate could act as enhancers of fruity aromas in red wines. Therefore, in order to further understand the role of volatile thiols in Carmenere red wine, gas chromatography-olfactometry and aroma extract dilution analysis experiments were conducted. Results showed the presence of 2-furanylmethanethiol, 3-sulfanyl-1-hexanol, 3-sulfanylhexyl acetate and 2-methyl-3-sulfanyl-1-butanol, the latter reported for the first time in red wines. Moreover, we showed that 3-sulfanyl-1-hexanol, with a dilution factor of 100, could be an important contributor to the Carmenere red wine aroma.

Finally, an extraction procedure and quantitation method to analyze in a single chromatographic run the five most important volatile thiols in wine was proposed and optimized. This method will provide a new tool to analyze and better understand the role of thiols in wine samples.

Keywords: Wine aromas, Carmenere, identification, quantitation, odorant compounds, sensory evaluation.

Members of the Doctoral Thesis Committee:

**Eduardo Agosin Trumper
Edmundo Bordeu Schwarze
Felipe Laurie Gleisner
Franco Pedreschi Plasencia
Ricardo Tapia Apati
Martin Steinhaus
Cristian Vial Edwards**

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ESCUELA DE INGENIERÍA

IDENTIFICACIÓN Y CARACTERIZACIÓN DE COMPUESTOS ODORANTES EN
VINOS CARMENERE

Tesis enviada a la Dirección de Investigación y Postgrado en cumplimiento parcial de los requisitos para el grado de Doctor en Ciencias de la Ingeniería.

CAROLINA PAVEZ MORENO

RESUMEN

El aroma de un vino es uno de los atributos más importantes que determinan la preferencia de los consumidores y por consiguiente, el mercado en el cual podrá ser comercializado y su precio final. Durante el año 2013, Chile se posiciona en el cuarto lugar entre los mayores países exportadores de vino por detrás de Francia, Italia y España. Según el último reporte de la Organización Internacional de la Vid y el Vino (OIV), las exportaciones Chilenas fueron cercanas a los US\$ 1,900 millones en 2013, con USA, Inglaterra y China como los mayores destinos de estas exportaciones.

Vitis Vinifera cv. Carmenere es una variedad de vino tinto originaria de la región de Bordeaux, Francia. Luego de la plaga de filoxera (*Dactylosphaera vitifoliae*) en Europa, se pensó que esta variedad Carmenere se había extinguido. Sin embargo, en 1994, ésta fue redescubierta en Chile, lugar donde en la actualidad se encuentran plantadas la mayor cantidad a nivel mundial de viñas de esta variedad. Sólo existen unos pocos estudios relacionados con los aromas que caracterizan la variedad de vinos Carmenere, los cuales han sido descritos con notas vegetales, especiadas y frutales. Los compuestos odorantes relacionados con los aromas vegetales, así como también, sus umbrales de percepción sensorial y valores de concentración, han sido bien abordados hasta ahora.

Sin embargo, existe escasa información sobre los compuestos odorantes relacionados con los aromas frutales y especiados.

Identificar cuáles son los principales compuestos odorantes relacionados con los aromas frutales en vinos Carmenere, es de gran importancia para poder elaborar los vinos de esta variedad según distintos estilos. Por lo tanto, el objetivo del presente trabajo de investigación fue identificar y caracterizar las principales zonas odorantes en un vino Carmenere, con especial énfasis en aquellos compuestos odorantes relacionados con los aromas frutales. Para este propósito, este estudio comenzó con un barrido de los compuestos odorantes más importantes, por medio del uso de cromatografía gaseosa acoplada a olfatometría y estudios de factores de dilución. Entre los odorantes identificados, se puso especial énfasis en los compuestos metil 2-metilbutanoato y metil 3-metilbutanoato, los cuales se reportan por primera vez en este trabajo en vinos tintos. El énfasis en estos compuestos resulta principalmente del hecho que podrían potenciar los aromas frutales en vinos tintos.

Adicionalmente, algunos estudios sobre tioles volátiles en vinos tintos reportaron que el compuesto 4-metil-4-sulfanil-2-pentanona podría ser el responsable del aroma a cassis, así como también que los compuestos 3-sulfanil-1-hexanol y 3-sulfanilhexil acetato podrían actuar como potenciadores de aromas frutales en vinos tintos. Por lo tanto, para entender mejor el rol de los tioles volátiles en vinos Carmenere se realizaron experimentos de cromatografía gaseosa acoplada a olfatometría y estudios de factores de dilución. Los resultados obtenidos muestran la presencia de 2-furanilmetanotiol, 3-sulfanil-1-hexanol, 3-sulfanilhexil acetato y 2-metil-3-sulfanil-1-butanol, este último reportado por primera vez en vinos Carmenere. Adicionalmente, el compuesto 3-sulfanil-1-hexanol con un factor de dilución de 100, podría contribuir de manera importante al aroma de los vinos Carmenere.

Por último, se propone la optimización para la extracción y cuantificación en una sola corrida cromatográfica, para analizar los cinco tioles volátiles más importantes en enología. El método propuesto proporciona una nueva herramienta para el análisis que

permitirá entender de mejor manera el rol que cumplen los tioles volátiles en muestras de vinos, así como también, todos los hallazgos realizados en el presente trabajo de investigación, contribuirán a aumentar el conocimiento acerca de los aromas en vinos Carmenere.

Palabras claves: Aromas del vino, Carmenere, identificación, cuantificación, compuestos odorantes, evaluación sensorial.

Miembros de la Comisión de Tesis Doctoral

Eduardo Agosin Trumper
Edmundo Bordeu Schwarze
Felipe Laurie Gleisner
Franco Pedreschi Plasencia
Ricardo Tapia Apati
Martin Steinhaus
Cristian Vial Edwards

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1 Introduction

1.1 Aroma compounds in foods and beverages

Foods and beverages contain volatile and non-volatile components, which by interaction with the human senses, cause the odor and taste sensations. The sum of taste and smell sensations evoked during consumption of a food substance is known as flavor (Belitz, Grosch, & Schieberle, 2009; Berger, 1995).

Aroma perception is set up by the detection of the volatile odorants by the olfactory receptor neurons located in the epithelium inside the nasal cavity (Swiegers, 2005). The human olfactory system is able to identify and distinguish between thousands of odorants with only approximately 400 functional odorant receptors. The latter are rhodopsin-like G protein-coupled seven trans membrane helix receptors. Interaction of the odorants with the receptors results in consecutive transduction events that initiate the opening of ion channels in neuron cell membranes; as a result, an action potential is generated and transmitted by the neuron to the olfactory bulb (Frings, 2001).

To recognize the odorants, the olfactory system uses a combinatorial receptor-coding scheme to encode odor identities. In this combinatorial scheme, one receptor has the ability to bind multiple odorants and individual odorants can stimulate several odor receptors, allowing the recognition of a large number of aromas. Additionally, there is a relationship between chemical structure of the odorants and odor quality. For example, a carboxylic acid and an aliphatic alcohol with the same carbon length chain have a strikingly different odor perception. Carboxylic acids are generally perceived as rancid, sour and sweaty; in contrast, aliphatic alcohols are perceived as herbal, floral and fruity (Malnic, Hirono, Sato, & Buck, 1999).

Flavor scientists have identified about 8000 volatile compounds in different food matrices, so far. However, only a small part of these compounds contribute to the overall aroma perception. Furthermore, only those present in a concentration above their odor threshold have the ability to contribute to the food aroma. The odor threshold is defined as the lowest concentration value that can be recognized by a representative human sensory panel (Belitz et al., 2009). A recent work proposed that only a group of 227 key

food odorants (KFO), in a combination of different concentration ranges, are responsible for the aroma of a wide range of food samples, such as alcoholic beverages, meat products, fish and seed oils, fruits, vegetables, mushrooms, spices and herbs, cocoa and chocolate, coffee, tea and some others including soy sauce, balsamic vinegar, honey and popcorn (Dunkel et al., 2014).

The origin of KFO in food could be divided in two groups, i.e. non-processed and processed food. In non-processed food, KFO are mainly produced by the fruit or vegetable metabolism. Smelling compounds, such as terpenes, belong to this group. On the other hand, for the processed food group, KFO are mainly produced by fermentation and thermal treatments (boiling, baking, cooking, roasting and frying). Compounds, such as furans generated by Strecker- and Maillard-type reaction and the compound 2-phenylethanol, synthesized by the yeast *Saccharomyces cerevisiae* during fermentation, belong to this second group (Dunkel et al., 2014).

An indicative parameter if a certain odorant compound could influence the overall aroma of a food sample is given by the ratio between its concentration value and its odor threshold, a concept known as odor activity value (OAV). Thus, an odorant present in a concentration well above its odor threshold could be a good candidate as an important contributor to the overall aroma of a food sample (Belitz et al., 2009). It is worth considering that odorants in food samples occur in nature as complex mixtures. Nevertheless, most of the odorants that have been isolated from natural food extract do not elicit aroma contributions that are reminiscent of its pure substance. For instance, butanoic acid is one of the major components of red wine aroma (Frank, Wollmann, Schieberle, & Hofmann, 2011). Although the pure substance has a strong cheese-like and rancid aroma descriptor, it confers a new odor perception to the overall red wine aroma, conveying a unique odor quality not elicited by the single components (Dunkel et al., 2014).

1.2 Wine aromas

Wine aroma has been described as slight sweet, pungent, alcoholic and a little bit fruity. Odorants that compose the wine aroma are mainly produced by the yeast metabolism during the alcoholic fermentation (Ferreira, Escudero, Campo, & Cacho, 2008). There are many factors that could influence the aroma composition of wine, e.g. environmental conditions (climate and soil), grape variety, degree of ripeness, fermentation conditions (pH, temperature and yeast flora), winemaking operations and aging (bottle or oaks maturation) (Rapp, 1998). However, understanding odorant composition alone is not sufficient to manage or predict the resulting wine aroma. This is due to the interactions that could occur between odorants and non-volatiles, including proteins, polysaccharides, lipids and polyphenols. These non-volatile compounds could be able to change the volatility of odorants and hence the overall aroma perception (Ebeler & Thorngate, 2009; Sáenz-Navajas et al., 2010).

1.2.1 Wine aroma classification.

In wines, aroma compounds are classified according to their origin, i.e. those synthesized by the grape berry metabolism and those produced during winemaking (Roland, Schneider, Razungles, & Cavelier, 2011).

1.2.2 Varietal aroma compounds. These aroma compounds are synthesized by the grape metabolism during berry growth and ripening. They could be present in their free form or as aroma precursors, linked to an amino acid or a sugar by a covalent bond. Even though the cleavage of linked aroma compounds occurs by yeast enzymatic action or acid hydrolysis, they belong to varietal aromas because they originate in the grape berry. The latter is the case for thiols, which will be discussed below.

1.2.2.1 Pre-fermentative aromas. These compounds are released between harvest and the onset of the alcoholic fermentation, particularly by enzymatic reactions during crushing. C₆ compounds (e.g. 1-hexanol, E-2-hexenal) belong to this group.

1.2.2.2 Fermentative aromas. The yeast and bacteria's metabolism synthesize these compounds during the alcoholic and malolactic fermentation, respectively. Examples of fermentative aromas are ethyl esters and fusel alcohols.

1.2.2.3 Post-fermentative aromas. These compounds are produced during wine aging in the bottle or oaks barrel, by means of chemical or biochemical reactions. These aroma compounds, such as vanillin or lactones give more complexity to the wine.

1.2.3 Aroma precursors in wine.

The typical wine aroma is mainly related to volatile compounds that arise from the grapes. However, in most fruits, including grapes, a significant part of these odorant compounds accumulate as non-volatile and odorless aroma precursors. These precursors could be linked to a sugar molecule (glycoside precursors) or an amino acid, such as cysteine or glutathione.

1.2.3.1 Glycoside precursors. Glycoside precursors are composed by an aglycone part, i.e. the volatile part that will be released, and a non-volatile sugar. Monoterpenes, C₁₃-norisoprenoids, benzene derivatives and long-chain aliphatic alcohols are among the aroma compounds present as aglycone. The sugar moiety includes glucose (O- β -D-glucosides) or disaccharides (O-diglycosides). In the case of diglycosides, generally carrying a β -1,4 linkage, the glucose moiety could include the following mono-saccharides: α -L-arabinofuranose, α -L-arabinopyranose, α -L-rhamnopyranose, β -D-apiofuranose, β -D-glucopyranose or β -D-xylopyranose. These odorless non-volatile glycosides are cleaved by acid or enzymatic hydrolysis occurring during grape-berry processing or wine storage, by mean acid hydrolysis reactions. In winemaking, acid hydrolysis reactions occur slowly and they are dependent of the pH, temperature and aglycone structure. On the other hand, glycosides may also be hydrolyzed by the action of β -glucoside glucohydrolases, commonly known as β -glucosidases (Pogorzelski & Wilkowska, 2007).

1.2.3.2 Thiol precursors. Strong smelling thiol compounds with odor thresholds in wine in the ng/L range are present in grapes as non-volatile precursors linked to cysteinylated or glutathionylated residues (Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2012). Volatile thiol compounds, such as 3-sulfanyl-1-hexanol (3SH), 3-sulfanylhexyl acetate (3SHA) and 4-methyl-4-sulfanyl-2-pentanone (4MSP), are the compounds predominantly responsible for fruity notes in several varietal white wines, particularly Sauvignon blanc (Tominaga, Furrer, Henry, & Dubourdieu, 1998). For red wines, 4MSP was described as compounds responsible for blackcurrant notes, whilst 3SH and 3SHA could act as an enhancer of fruity aromas in this kind of wines (Rigou, Triay, & Razungles, 2014). However, the role of volatile thiol compounds in red wine has not been clearly elucidated, so far. Tominaga et al., 1998 were the first to describe the cysteinylated thiol as precursors in Sauvignon blanc grapes. There are cysteinylated precursors of the compounds 3SH, 4MSP and 4-methyl-4-sulfanyl-2-pentanol (4MSPOH). Furthermore, wines elaborated from *Botrytis cinerea* infected grape berries were recently found to contain the cysteinyl precursors for 3-sulfanyl-1-pentanol (3SP), 3-sulfanyl-1-heptanol (3SHp) and 2-methyl-3-sulfanyl-1-butanol (2M3SB) (Sarrazin et al., 2007). Volatile thiols linked to cysteine are released by the enzymatic action of a yeast β -lyase. This enzyme cleaves the thioether bond of L-cysteine conjugates (S-alkyl and S-aryl). This cleavage is specific for the carbon–sulfur bond (Tominaga, Murat, & Dubourdieu, 1998).

Glutathionyl linked 3SH and 4MSP were discovered later (Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009). Volatile thiols from glutathion adducts are released in the same way as from cysteine adducts. However, the cysteine adduct of 3SH could also derive from the catabolism of the respective glutathionyl adduct. This reaction needs the presence of two enzymes: a γ -glutamyltranspeptidase, which catalyzes the removal of glutamic acid and a carboxypeptidase, which removes glycine in order to form 3SH-cysteine (3SH-cys) (Roland, Schneider, Charrier, et al., 2011). Additionally, the synthesis of 3SH-cys in grapevine cells results from the cleavage of 3SH-glutathione

(3SH-glu), which is spontaneously formed after the conjugation of glutathione to E-2-hexenal (Roland, Schneider, Razungles, et al., 2011; Thibon et al., 2010).

1.2.4 Major wine odorant compounds.

As described above, wine aroma is derived from a complex mixture of different odorants, which are either produced by the varietal grape berry, microbial fermentation or wine aging. Ferreira et al., 2008 introduced the concept of wine matrix aroma or basal wine aroma. This vinous aroma is common for all wine varieties and is caused by ethanol and the major aroma-active metabolites of fermentation, such as fusel alcohols (e.g. 2-and 3-methyl-1-butanol), ethyl esters (e.g. ethyl hexanoate, ethyl butanoate), alcohol acetates (e.g. hexylacetate, 3-methylbutyl acetate), acetoin (3-hydroxy-2-butanone) and acetaldehyde. The result of the combination of these odorants gives the characteristic alcoholic, pungent, sweet and a little bit fruity basal wine aroma.

Additionally, Ferreira postulates that the basal wine aroma plays a buffer role, defined as the ability to counteract the effect of the omission from the mixture of one of its components and the ability to counteract the addition of many single odorants to the mixture. Sensory experiments, performed in a dearomatized white wine from Maccabeo variety, supports the hypothesis of the wine aroma buffer (Escudero, Gogorza, & Melus, 2004). The authors found that only when β -damascenone was omitted, panelists were able to perceive a change in the overall aroma of the wine. On the other hand, for the addition tests, only 3-methylbutyl acetate (isoamyl acetate) and γ -nonanolactone, of the 13 odorants assessed, produced a change in the overall Maccabeo wine aroma. Panelists reported an increase in the banana-like, floral, and citric aroma nuances when the natural amount of these components in the wine was tripled (Escudero et al., 2004).

According to Ferreira et al., 2008 the wine aroma buffer could be broken in 4 different ways:

- By the interaction of a high impact compound, such as rotundone or cis-rose oxide in Shiraz and Gewürztraminer wines, respectively (Guth, 1997; Parker, Pollnitz, Cozzolino, Francis, & Herderich, 2007). These

impact compounds could influence and change the overall wine aroma, without the need of the support of additional odorants.

- By the concerted action of some chemical families that share similar chemical structures and odor qualities, such as γ -lactones and ethyl esters.
- By the action of subtle compounds. These compounds are not able to impart their aroma nuance without a support of other odorants with similar aroma characteristics. Nevertheless, if subtle compounds fail in transmitting their specific aroma nuances to the wine, they contribute decisively to the development of some secondary-generic aroma nuance (e.g. fruity and sweet-like aromas)
- By the action of odorant enhancers. These compounds could be found in all wine varieties, however in certain concentration they could change the overall wine aroma by enhance some fruity aromas; such is the case of β -damascenone and dimethyl sulphide (DMS) (Escudero, Campo, Fariña, Cacho, & Ferreira, 2007; Pineau, Barbe, Van Leeuwen, & Dubourdieu, 2007).

Clearly, wine is a quite complex matrix and the wine aroma is the result of a combination of at least 28 odorants that in a certain concentration results in the distinctive aroma (Frank et al., 2011). Some of the major odorants present in wine are the following:

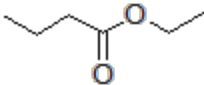
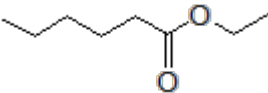
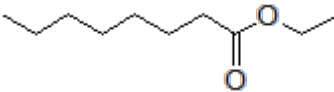
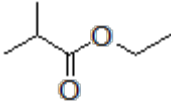
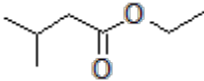
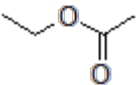
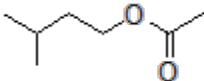
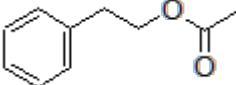
- **Ethyl esters and acetates.** These compounds are mainly responsible for the fruity aroma of wines (Lytra, Tempere, de Revel, & Barbe, 2012). Ethyl esters are mostly produced by yeast cells through lipid and acetyl-CoA metabolism (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Meanwhile, the synthesis of acetate esters of fusel alcohols by *Saccharomyces cerevisiae* has been referred to the activity of at least three acetyl-transferases (AAT, EC 2.3.1.84), namely alcohol acetyl-transferase, ethanol acetyl-transferase and isoamyl alcohol acetyl-

transferase (Plata, Millan, Mauricio, & Ortega, 2003). Ethyl esters and acetates are often found in concentrations above their odor thresholds (Jackson, 2008). The most abundant of the latter compounds in wine is ethyl acetate. This compound, in concentrations below 80 mg/L could have a positive effect to the wine aroma. However, in concentrations above 80 mg/L, it is considered an off-flavor due to its varnish-like aroma. Some of the major ethyl esters and acetates are listed in Table 1.1.

- **Thiols.** As described above, thiol compounds are found as odorless precursors in grape berries. They are linked to cysteine or glutathione. During alcoholic fermentation, the yeast releases them by the β -lyase enzymatic activity (Roland, Schneider, Razungles, et al., 2011). Thiol compounds have been widely studied in white wines and they are mainly responsible for the exotic fruit odor note in Sauvignon blanc wines. They are characterized by their extremely low odor detection threshold, in the range of ng/L, as determined in a synthetic wine model solution (10% v/v ethanol, pH 3.5). Therefore, they are able to influence the overall wine aroma in quite small concentrations.

The most abundant aroma-active thiols found in wines are 3-sulfanyl-1-hexanol (3SH), 3-sulfanylhexyl acetate (3SHA), and 4-methyl-4-sulfanyl-2-pentanone (4MSP). These thiol compounds are associated with different sensory descriptors, such as grapefruit, passion fruit and boj tree. Other important aroma-active thiols in wines are 2-furanylmethanethiol (FFT) and phenylmethanethiol (PhMT). FFT is responsible for roasted coffee or toasty aroma notes and is mainly released by oak barrels during aging (Blanchard, Tominaga, & Dubourdieu, 2001). PhMT is responsible for smoky aroma notes and its origin in wines is unclear (Tominaga, Guimbertau, & Dubourdieu, 2003). Odor qualities, structures and odor thresholds of these thiol compounds are summarized in Table 1.2.

Table 1.1. Major ethyl esters and acetates commonly found in wines.

Compound	Odor quality	Structure	Odor Threshold ^a ($\mu\text{g/L}$)
Ethyl butanoate	Pineapple		20 ^b
Ethyl hexanoate	Apple		14 ^c
Ethyl octanoate	Brandy pear		5 ^b
Ethyl 2-methylpropanoate	Fruity		15 ^d
Ethyl 3-methylbutanoate	Fruity, apple		3 ^e
Ethyl acetate	Varnish		12270 ^b
3-methylbutyl acetate	Banana		30 ^c
2-phenylethyl acetate	Honey		250 ^b

^a In a synthetic wine solution (10% v/v ethanol, pH 3.2).

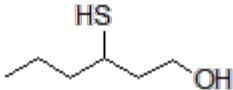
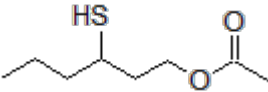
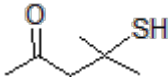
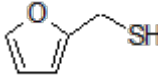
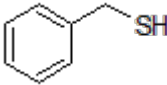
^b Escudero et al., 2007.

^c Francis and Newton, 2005.

^d Ong et al., 1999.

^e Cullere et al., 2004.

Table 1.2. Most widely studied aroma-active thiols in wine.

Compound	Odor quality	Structure	Odor Threshold ^a (ng/L)
3-sulfanyl-1-hexanol	Grapefruit		60 ^b
3-sulfanylhexyl acetate	Passion fruit		4.2 ^b
4-methyl-4-sulfanyl-2-pentanone	Box tree, blackcurrant bud		0.8
2-furanylmethanethiol	Coffee, roasted		0.4
Phenylmethanethiol	Smoky		0.3

^a Odor threshold determined in a synthetic wine solution (10% v/v ethanol, pH 3.5), according to Roland et al., 2011.

^b Odor threshold of the racemic mixture.

- **Methoxypyrazines.** Methoxypyrazines are varietal compounds synthesized by the grape berry during early developmental stages. They reach a maximum concentration level before veraison. Veraison is the onset of the ripening and is the moment when grape berries change their color.

There are two postulates about the biosynthesis pathway of 3-alkyl-2-methoxypyrazines, commonly found in grapes. The first postulate suggests the condensation of an α -amino acid amide, such as valine, with glyoxal to produce 3-alkyl-2-hydroxypyrazine. The second postulate proposes valine and glycine as building blocks to produce 3-alkyl-2-hydroxypyrazine. The final step of both postulates involves the methylation of 3-alkyl-2-hydroxypyrazine mediated by an *O*-methyltransferase in the presence of S-adenosyl-L-methionine as the methyl donor group (Dickschat et al., 2011; Vallarino et al., 2011).

Methoxypyrazines are mainly responsible for the vegetal aromas, such as bell pepper and green beans (Belancic & Agosin, 2007). In grapes, three types of methoxypyrazines have been identified (Figure 1.1), the most abundant being 3-isobutyl-2-methoxypyrazine (IBMP) with a low odor detection threshold in wine (15 ng/L). Concentration values of methoxypyrazines in wines are quite variable, ranging from 0.5 to 60 ng/L (Catania and Avagnina, 2010).

The concentration levels of methoxypyrazines decrease when the grape berry ripens, as well as with the sun exposition (Belancic & Agosin, 2007). Therefore, in terroirs with warm climate, low concentrations of methoxypyrazines in the resulting wines are expected. Additionally, viticultural practices, such as partial leaf removal, could also yield low concentrations of these compounds in resulting wines.

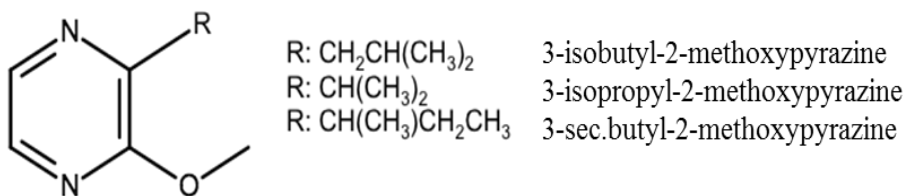


Figure 1.1. Chemical structures of the methoxypyrazines commonly found in grapes.

- Terpenes and C₁₃-norisoprenoids.** Terpenes are secondary metabolites in fruits. The synthesis of terpenes in plants involves the formation of isopentenyl pyrophosphate by means of successive phosphorylation reactions from β -hydroxy- β -methylglutaryl coenzyme A (Charlwood, Banthorpe, & Francis, 1978). Meanwhile, C₁₃-norisoprenoids are produced by oxidative degradation of carotenoids. Both compound groups reach their maximum concentration at the end of the grape ripening, as well as terpenyl - glycosides. Must concentrations for the latter, ranging from 500 to 1700 $\mu\text{g/L}$, have been reported (Villena, Iranzo, & Pérez, 2007). Terpenes and C₁₃-norisoprenoids are released from their glycoside precursors by chemical or enzymatic hydrolysis during winemaking and wine aging. Acid hydrolysis of these compounds occurs quite slowly under winemaking conditions and is closely dependent on the pH conditions, temperature and structure of the aglycone moiety (Pogorzelski & Wilkowska, 2007). In a synthetic wine solution (10% v/v ethanol, pH 3.2), terpenes occurring in aroma-active amounts in wine have odor threshold values in the order of $\mu\text{g/L}$; whilst such C₁₃-norisoprenoids thresholds are in the order of ng/L (Escudero et al., 2007). These compounds together give more complexity to the wines, providing flowery and fruity aroma nuances.
- C₆-compounds.** These compounds are produced by enzymatic decomposition of unsaturated fatty acids, mainly during grape crushing. They provide vegetal and herbaceous odor notes to the wine (Pisarnitskii, 2001).

- **Fusel alcohols.** These compounds are originated from *Saccharomyces cerevisiae* metabolism via the Ehrlich pathway and derived from amino acid catabolism. Amino acids that are assimilated by the Ehrlich pathway are valine, leucine, isoleucine, methionine and phenylalanine and they are slowly converted throughout the fermentation. The initial step of Ehrlich pathway is a transamination reaction resulting in a α -keto acid intermediate, which can subsequently be decarboxylated into an aldehyde and finally reduced to the fusel alcohol (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). Odor threshold values for fusel alcohols vary between 19 mg/L for 2-methyl-1-propanol to 36 μ g/L for 3-methylsulfanyl-1-propanol (methionol) and they could confer aroma nuances such as sweet and malty (2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol), sweet and flowery (2-phenylethanol) and cooked potato (methionol) (Styger, Prior, & Bauer, 2011).
- **Odorant compounds derived from oak wood.** Certain odorant compounds are extracted from oak barrels during wine aging. These compounds could originate by means of different pathways, such as oxidation reactions, lignin or hemicellulose degradation or microbial synthesis. Some of the oak derived compounds are lactones (e.g. 5 butyldihydro-4-methyl-2-(3H)-furanone, known as whisky lactone), vanillin (4-hydroxy-3-methoxybenzaldehyde) and other volatile phenols (e.g. 2-methoxyphenol). These compounds give more complexity to the wine, providing odor notes like vanilla, coconut, clove-like and leather, among others (Pérez-Prieto, López-Roca, Martínez-Cutillas, Pardo-Mínguez, & Gómez-Plaza, 2003).
- **Off-flavors.** Aroma-active compounds able to decrease the positive sensory characteristics of the wine or to produce an aromatic unbalance that diminish the wine quality are known as off-flavors compounds. This concept is, however, dependent on wine consumer preferences and expectations and, therefore, strongly influenced by of previous knowledge and experience with a certain wine variety (Ferreira, Escudero, Campo, & Cacho, 2008).

There are some microbial generated off-flavors that change the overall aroma of the wine. For example, volatile phenols are produced by the yeast enzyme cinnamate decarboxylase. This enzyme decarboxylates cinnamic acid derivative, releasing volatile phenols, such as 4-vinylphenol and 2-methoxy-4-vinylphenol (4-vinylguaiacol) with phenolic and pharmaceutical aroma nuances. The odor detection threshold of both compounds is close to 700 ng/L (Catania and Avagnina, 2010). Other yeast-related off-flavor compounds are 4-ethylphenol and 2-methoxy-4-ethylphenol (4-ethylguaiacol) produced by the genus *Brettanomyces/Dekkera* (Dias et al., 2003). These compounds are responsible of the horse-like, leathery notes in wines with odor detection thresholds of 440 µg/L for 4-ethylphenol and 33 µg/L for 4-ethylguaiacol (Escudero et al., 2007).

1.2.5 Aromas in Carmenere red wine.

Vitis Vinifera. Carmenere is a red grape variety originating from the Bordeaux region, France. Carmenere was thought to be extinct after the phylloxera plague in Europe, but in 1994, it was rediscovered in Chile, where most of the worldwide vineyards of this grape variety are currently planted. Carmenere grape berries have a medium size (1,3 – 1,6 g) with a high potential to sugar accumulation (Pszczółkowski, 2004).

A descriptive sensory map of Carmenere red wine was developed by Casaubon et al. 2006. Samples were selected in order to cover the edaphoclimatic diversity in Chile. For this purpose, different terroirs among valleys from Limarí (IV region) to Bio-Bio (VIII region), were included, as well as locations with different altitude and proximity to the sea. Sensory descriptors were determined by free choice profiling with an expert sensory panel. Statistical analysis of the data was performed by application of principal component analysis (PCA), characterizing the Carmenere sensory space as vegetable, spicy, and fruity smelling (Figure 1.2) (Casaubon, Belancic, & Agosín, 2006).

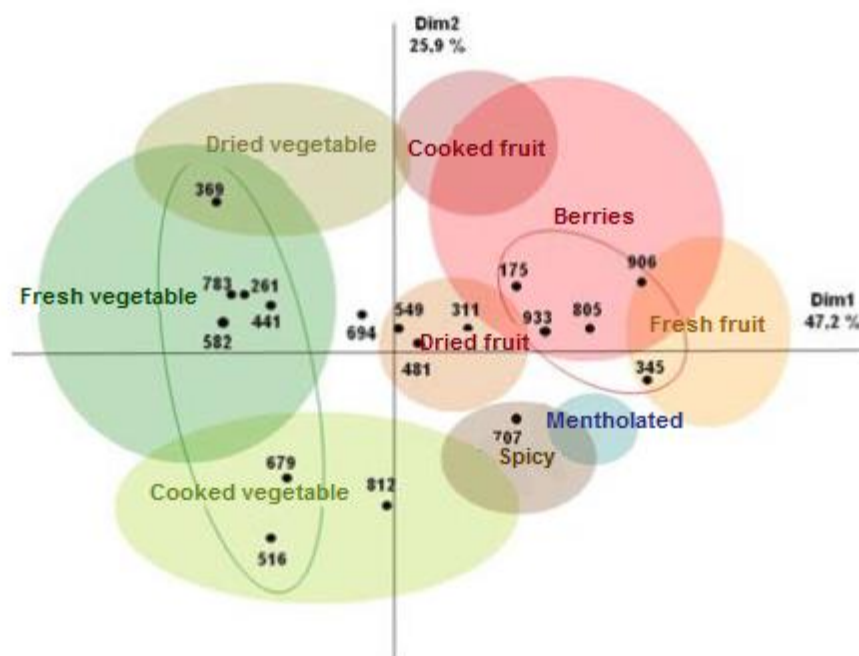


Figure 1.2. Sensory descriptive map of the Carmenere red wine samples, as analyzed by an expert panel. Three digit codes represent the samples analyzed. The graph explains a 73% of the variability of the whole samples and mainly characterizes the Carmenere red wines as vegetable, spicy and fruity smelling (Casaubon et al., 2006).

Aroma profiles of Carmenere wines are strongly dependent on the time of grape harvest. One of the main features of Carmenere grape berries is their requirement for late harvest to enhance fruity aromas in detriment of vegetable nuances. However, this practice should be conducted carefully to avoid the occurrence of fungal infection by *Botrytis cinerea*, for example (Pszczółkowski, 2004). Another viticultural practice commonly used to increase the fruity aroma note is the canopy management and leaf removing (Fredes, Moreno, Ortega, & Von Bennewitz, 2010).

Vegetable-like aroma notes of Carmenere are mainly related to methoxypyrazines (3-isobutyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine). Methoxypyrazines (MP) are synthesized from the degradation of certain amino acids by the grape berry

metabolism. MP concentrations in Carmenere range between 2 and 45 ng/L. The orthonasal recognition threshold of 3-isobutyl-2-methoxypyrazine (IBMP) in red wines is 15 ng/L. Wines with concentration values of IBMP above 30 ng/L have strong vegetal aromas notes, such as bell pepper-like and green bean-like. Additionally, it was also reported that MPs are strongly dependent on the ripening level of grapes, sun exposition, rain events and terroir. Low concentrations of these compounds are associated with high sun exposition and overripening (Belancic & Agosin, 2007).

On the other hand, fruity and spicy aromas in Carmenere red wines are mostly related to C₁₃-norisoprenoids produced by hydrolysis of their glycoside precursors, mostly during wine aging. The most important C₁₃-norisoprenoids found in Carmenere that could enhance fruity odor notes are hydroxy- β -damascone, 3-oxo- α -ionol and 3-hydroxy- β -ionone. The concentration of their glycosidic forms range between 1500 to 7500 mg/L. C₁₃-norisoprenoids concentration reached a maximum close to grape ripening and decreased with overripening. Additionally, an inverse relationship between MP concentration and C₁₃-norisoprenoid precursor concentration has also been reported. In other words, a higher MP concentration in grape berries is associated with a lower concentration of C₁₃-norisoprenoid precursors and vice versa (Belancic & Agosin, 2007a)

The concentration of these compounds in their free forms ranged from 100 – 500 μ g/L (Belancic & Agosin, 2007a). This low conversion ratio could be explained by the occurrence of a slow hydrolytic reaction of the glycosidic precursors under winemaking conditions, i.e. pH and temperature; the chemical structure of the C₁₃-norisoprenoid precursor could also be of importance (Pogorzelski & Wilkowska, 2007).

Dominguez and Agosin, 2010 reported the presence of 3SH, 3SHA, PhMT and FFT in Carmenere red wine samples from Colchagua and Maipo Valleys. The authors applied a specific work-up extraction procedure for thiol compounds by an anionic exchange resin, and found the following average concentrations: 10.1 ng/L (FFT), 14.1 ng/L (PhMT), 666.8 ng/L (3SH) and 373.4 ng/L (3SHA). All concentration values were above the odor detection thresholds previously reported in synthetic wine solution for all

the thiol compounds analyzed (Roland, Schneider, Razungles, et al., 2011). This study provided the first quantitative information of aroma-active thiols in Carmenere red wine. However, the sensory impact of these compounds was not addressed. Based on the results obtained by Dominguez and Agosin, 2010, the present thesis further explored the role of thiols compounds in Carmenere wines by application of aroma extract dilution analysis (AEDA) and GC-olfactometry experiments as well as the optimization of the extraction and quantitation procedures for these compounds.

1.3 Isolation of volatile compounds from foods and beverages.

Aroma compounds are volatile molecules with low molecular weight, which, at a certain concentration, interact with the human odor receptors. For this reason, the extraction of volatile molecules is an important step in the elucidation of aroma-active compounds in a food sample.

Isolation of volatiles must be carried out using gentle methods that avoid artifact generation. Aroma artifacts could be produced by enzyme-catalyzed reactions, thermal degradation and oxido-reduction reactions. The most important isolation methods for volatile compounds in foods and beverages are distillation and headspace analysis.

1.3.1 Distillation

Distillation is a physical process for separating the components from a liquid mixture by evaporation and further condensation of the vapor obtained. The efficiency of the distillation is determined by the volatility of the compounds in the mixture. Distillation is a common procedure employed to isolate volatile from non-volatile components from a food matrix.

Distillation devices consist normally of a round-bottom flask for introducing the sample. By heating, highly volatile compounds evaporate and are collected in a second flask by condensation of the vapor by means of a cooling device. Distillation is a fast and versatile technique; however, application of temperatures above 50°C could promote degradation of some volatile compounds and the production of artifacts.

Solvent Assisted Flavor Evaporation (SAFE) is a high vacuum, short path distillation device, used for gentle isolation of aroma substances from food extracts by means of distillation (Engel, Bahr, & Schieberle, 1999).

1.3.2 Headspace analysis.

A simple procedure to analyze the volatile components in a food is headspace analysis. This analysis could be performed by introducing the sample in a sealed container and leaving it until the equilibrium between the matrix and the vapor phase is reached. There are three major headspace methodologies currently applied:

- **Static headspace** is the classical and the simplest headspace technique. The sample is taken from the vapor above the sample with a gas-tight syringe.
- **Dynamic headspace** is a technique which uses a flow of carrier gas through the sample vessel to increase the headspace sample size, and thus the sensitivity of the technique. Volatile compounds are isolated by passing through the sample an inert gas (e.g. nitrogen, helium) and then trapping the stripped compounds in the gas in a porous resin.
- **Solid Phase Micro Extraction (SPME).** Method consisting of the adsorption of volatiles on a fiber coated with an appropriate stationary phase (e.g. non-polar polydimethylsiloxane, polar polyacrylate or both combinations). The volatiles retained on the fiber are stepwise desorbed with a temperature gradient into a gas chromatograph injection port. This method saves preparation time, solvent usage and disposal costs. The detection limit of the analyte under study could be improved, as well (Belitz et al., 2009; Kataoka, Lord, & Pawliszyn, 2000).

1.4 Identification and quantitation of odorant compounds in foods and beverages.

After volatile extraction, the compounds must be separated and identified. Separation is carried out by means of gas chromatography, using capillary columns with different

polarities to improve compound resolution. The first step includes the identification of odorant compounds by locating the main odorant zones in the chromatogram. The second step correlates each odorant zone with a chemical structure, by comparing parameters such as retention indices, mass spectrum and odor qualities with a reference compound and finally in a third step the quantitation of the odorants is performed.

1.4.1 Gas chromatography-olfactometry (GC-O).

Not all the volatile compounds extracted from a food or beverages contribute to its overall aroma. Only some, in concentrations above their odor threshold, could interact with the human receptors and be perceived as aroma.

The application of gas chromatography-olfactometry (GC-O) in food flavor analysis represents a valuable technique to characterize odor-active compounds in a food sample. GC-O is an analytical technique able to precisely describe and characterize stimuli, evaluating and measuring impressions. GC-O is also a powerful analytical tool for the comprehension and quantification of a sensorial characteristic (d'Acampora Zellner, Dugo, Dugo, & Mondello, 2008).

For this purpose, at the end of the capillary column in the GC, the flow is split for being analyzed in two different detectors. One of the detectors, which produces the chromatographic signal, is a flame ionization detector (FID) or a mass spectrometer - detector. The other detector is a sniffing port adapted for the human nose olfaction (Belitz et al., 2009). This arrangement makes GC-O a unique technique that combines an analytical signal with the selectivity and sensitivity of the human nose.

1.4.2 Aroma Extract Dilution Analysis (AEDA).

AEDA consists of a series of GC-O analyses applied to the stepwise dilutions of the food extract until no odors are perceived anymore at the sniffing port by the panelists. Odorants that reach the highest flavor dilution factors (FD) are the main candidates to be key odor compounds of the food sample under analysis. Results of AEDA are presented

in an aromagram that correlates the resulting FD values and the retention indices of the odorants (Guth & Grosch, 1994).

1.4.3 Quantitation of odorant compounds.

Once the major odorants have been identified, the next step in food aroma analysis is to quantitate their amount in the sample under analysis. The main analytical technique used for this purpose - due to its sensitivity that makes possible detection at trace levels - is gas chromatography coupled to mass spectrometry (GC-MS).

The analytical method used for quantitation of the odorants should be suitable for the food matrix, repeatable, reproducible and with sensitivity or detection limits below the odor threshold of the odorants in the food matrix studied. In order to avoid and correct the experimental losses associated to the sample extraction procedure, addition of an internal standard at a known concentration is strongly recommended. Internal standard should have the same or closely similar chemical behavior as the analyte (Guth & Grosch, 1993). The best internal standard would be the same analyte labeled with an appropriate isotope, provided that the labelling is stable during the analytical procedure. This method is known as Stable Isotope Dilution Assay (SIDA) (Schieberle & Grosch, 1987).

1.4.4 Sensory evaluation of an aroma model based on the previously determined odorant concentration.

Sensory evaluation is defined as a scientific discipline used to evoke, measure, analyze, and interpret those responses to products that are perceived by the senses (Sidel & Stone, 1993).

In food aroma research, sensory evaluation is the last step to validate all the data obtained by GC-O, AEDA and quantitation experiments. One of the main sensory analyses applied to obtain the aroma profile of a food sample is the descriptive sensory analysis. This analysis involves the detection (discrimination) and description of both

the qualitative and quantitative sensory components of a consumer product by trained panels of judges (Murray, Delahunty, & Baxter, 2001).

To evaluate the impact of a certain odorant, addition or omission sensory tests are commonly carried out. The aim of these tests is to evaluate the sensory differences between the sample under study with or without the odorant. The most important sensory tool to assess differences between two food samples is the discriminate triangular test. In the latter, the panelists have to assign the different sample by a forced-choice rule. Analysis of the data is processed statistically by a binomial distribution, according to the number of right answers (ISO 4120:2004).

1.5 Thesis motivation.

Odorant compounds are critical for wine quality, because they determine the category and level of acceptance of the consumers. The market segments, as well as the final price of a wine are also significantly related with the aroma, - particularly for white wines.

The Chilean wine industry is one of the most important worldwide with excellent quality products. Among the major wine export countries, Chile ranks fourth behind France, Italy and Spain. According to the last report of the International Organization of Vine and Wine (OIV), Chilean wine exports were close to US\$ 1,900 million in 2013 with USA, England and China as the major export destinations.

According to the Agricultural and Livestock Service of the Chilean Government (SAG), until December of 2013, Chile has a total cultivated vineyard surface of 130,361.7 ha. The relative cultivated surfaces of the different wine grape varieties are Cabernet sauvignon (32.3%), Sauvignon blanc (11.0%), Merlot (9.1%), Chardonnay (8.2%) and Carmenere (8.1%). Among the Chilean red wine varieties, Carmenere is the third most important grape variety cultivated, behind Cabernet Sauvignon and Merlot. It is worth to mention that almost all worldwide Carmenere vineyards are cultivated in Chile. Therefore, the latter is a distinctive grape variety of the Chilean winemaking industry.

Unfortunately, there is scarce information about the key odorant compounds that characterize the Carmenere aroma.

So far, only the odorant compounds related to herbaceous aromas have been characterized, as well as the influence of the terroir and winemaking process in their expression into the wine. To the best of our knowledge, no information is available on the key chemical compounds related with the fruity/spicy aromas of Carmenere red wines, which correspond to the main sensory descriptors of this variety - together with vegetable aromas - and among the most appreciated by wine consumers.

1.6 Hypothesis

The following hypotheses support this research:

- i. The overall aroma of Carmenere red wines could be differentiated by the odorant compounds that are mainly synthesized by the Carmenere grape berry during ripening.
- ii. The characteristic fruity aroma of Carmenere red wines results by the presence of few odorant impact compounds, which in concentrations above their odor thresholds, are able to impart their typical odor nuances.
- iii. The Carmenere red wine aroma could be influenced by a synergistic effect of some odorants, which could share a similar chemical structure, as well as a similar odor nuance.
- iv. Thiols are key compounds for the aroma quality of several white wines. However, they have been scarcely characterized in red wines. We hypothesize that in Carmenere wines the fruity descriptors commonly employed to describe this variety could be related with this type of compounds.

1.7 Objectives

The general objective of this research was to contribute to the identification of the main odorant compounds of Chilean Carmenere red wine.

Specific objectives were:

- i. Identification of the most important odorant zones in Chilean Carmenere red wine by means of gas chromatography olfactometry and Aroma Extract Dilution Analysis.
- ii. Identification of potential new odorant compounds and evaluation of their sensory contribution to Carmenere red wine aroma.
- iii. Identification of major thiol compounds present in Carmenere red wine and assessment of their impact on the aroma of the variety.
- iv. Development of a novel extraction procedure and analytical method for simultaneous identification and quantitation of the major thiol compounds in wine samples.

1.8 Content and contribution

The research about Carmenere red wine aromas began with a GC-O analysis, to locate the most important odorants, followed by AEDA experiments to find out those with the highest flavor dilution (FD) factors in a Carmenere red wine from Peumo, Cachapoal valley, a well-recognized region for producing high quality Carmenere red wines with fruity and spicy aromas.

The second step of this work was to obtain a map of some Carmenere red wines, in order to evaluate the differences between commercial Carmenere from different wineries.

By application of GC-O and AEDA analyses, 21 odorant compounds with a $FD \geq 16$ were identified. Most of the odorants were addressed as constituents of red wine matrix. However, one odorant zone was identified as a mixture of the compounds methyl 2-methylbutanoate and methyl 3-methylbutanoate. These compounds were then quantitated in the Carmenere red wine screened by AEDA and another 14 red wine varieties, and finally their odor activity values were determined.

Thiol compounds in red wine have been reported to be responsible of blackcurrant aromas and as enhancers of fruitiness, as well. Therefore, we carried out a specific thiol extraction work-up by using a mercurated agarose resin. The resulting extract was submitted to GC-O and AEDA. Four odorant zones could be identified as 2-

furanylmethanethiol (FFT), 3-sulfanyl-1-hexanol (3SH), 3-sulfanylhexyl acetate (3SHA) and 2-methyl-3-sulfanyl-1-butanol (2M3SB), the latter was identified for the first time in red wines.

Finally, we developed a methodology to extract and quantitate the five most important polyfunctional thiols (FFT, 3SH, 3SHA, 4-methyl-4-sulfanyl-2-pentanone and phenylmethanethiol) in wines in a single chromatographic run.

2. Identification of odor-active compounds in red wines of *Vitis vinifera* Carmenere by aroma extract dilution analysis and gas chromatography-olfactometry.

Vitis Vinifera Carmenere is a red grape variety that produces wines with characteristic vegetable, spicy and fruity aroma notes. Vegetable aromas in this wine variety are mainly related to alkyl-methoxypyrazines (3-isobutyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine) (Belancic & Agosin, 2007). On the other hand, fruity and spicy aromas have been partially correlated with C₁₃-norisoprenoids released by hydrolysis from corresponding glycoside precursors (Belancic & Agosin, 2007a). However, no information exists about the main odorants related to Carmenere red wine and which of these characterize its overall aroma. Therefore, the aim of this study was to identify the main odorants in a Carmenere red wine by means of gas chromatography-olfactometry (GC-O) and aroma extract dilution analysis (AEDA).

2.1. Materials and methods.

2.1.1. Wine samples

Odorant profiling was carried out in a 100% varietal Carmenere red wine of Anakena winery (harvest 2011), produced at Las Cabras, Sixth Region, Chile.

2.1.2. Chemicals

The following reference odorants were obtained from Sigma-Aldrich Chemie, Taufkirchen, Germany: 4-allyl-2-methoxyphenol (eugenol), butanoic acid, 5-butyl-4-methyldihydro-2(3*H*)-furanone (whisky lactone), ethyl butanoate, ethyl cinnamate, ethyl hexanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 2-methylpropanoate, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolon), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 2-methoxyphenol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methylbutanoic acid, 3-methylbutanoic acid, methyl 2-methylbutanoate (M2MB), methyl 3-methylbutanoate (M3MB), 3-(methylthio)propanal and 3-(methylthio)-1-propanol. (*E*)- β -Damascenone was a gift from Symrise, Holzminden, Germany.

2.1.3. Isolation of volatiles

100 mL of the wine was first saturated with sodium chloride. The mixture was then extracted with diethyl ether (3×100 mL) and the combined organic phases were dried over anhydrous sodium sulfate. The volatiles were isolated by solvent-assisted flavor evaporation (SAFE) at 40 °C (Engel et al., 1999). The SAFE distillate was separated into acidic (AV) and neutral/basic volatiles (NBV) by acid/base extraction. For this purpose, the SAFE distillate was treated with aqueous sodium carbonate (0.5 mol/L, 3×50 mL); the organic phase contained the NBV. The combined aqueous layers were adjusted to pH 2.5 with hydrochloric acid (16% v/v in water) and extracted with diethyl ether (3×100 mL) to obtain the AV. The resulting fractions were concentrated (1 mL) using a Vigreux column (50×1 cm).

2.1.4. High resolution gas chromatography-olfactometry (HRGC-O) and high resolution gas chromatography-mass spectrometry (HRGC-MS).

HRGC-O was carried out with a 5160 Mega Series gas chromatograph (Carlo Erba Instruments; Milano, Italy), using FFAP and DB-5 fused silica capillaries (both $30 \text{ m} \times 0.32 \text{ mm id}$, $0.25 \text{ }\mu\text{m}$ film thickness; J&W Scientific, Agilent Technologies, Waldbronn, Germany). Samples were introduced into the GC by the cold-on-column injection technique at 40 °C and using helium as carrier gas. The oven programs and the carrier gas flow rates were the same as described by Frank et al., 2011. At the end of the capillary column, the effluent was split 1:1 by volume using a Y-shaped glass splitter and two deactivated fused silica capillaries ($50 \text{ cm} \times 0.20 \text{ mm id}$). One part was directed into an FID detector and the other part into a tailor-made sniffing port, kept at 220 °C. Calculations of linear retention indices (RI) were carried out after co-injection with a series of *n*-alkanes, as described previously analyses.

Aroma profiling of 4 Carmenere red wines was performed by quantitative descriptive analysis. Evaluation of the Carmenere red wine samples was made by eighteen panelists trained in sensory analysis and recruited from the German Research Centre for Food Chemistry. Odorants chosen for carrying out the aroma profile are those reported as the

main constituents of red wine aroma (Frank et al., 2011). The training of the panelist was performed in weekly sessions using aqueous solutions of the following reference compounds, in defined concentrations above their orthonasal odor detection thresholds. Flowery (2-phenylethanol; 72000 µg/L), malty (2-methyl-1-butanol; 71000 µg/L), fruity (ethyl hexanoate; 280 µg/L), cooked apple (β-damascenone; 8 µg/L), clove-like (eugenol; 270 µg/L), sweaty (butanoic acid; 1250 µg/L), smoky (2-methoxyphenol; 350 µg/L), vanilla-like (vanillin; 150 µg/L), coconut-like (γ-nonalactone; 390 µg/L), butter-like (2,3-butanedione; 1850 µg/L) and cooked potato-like (3-(methylthio)propanal; 300 µg/L). All samples (20 mL) were presented into PTFE vessels with cap. The panelists evaluated the intensity of each aroma descriptor on a seven-point scale from 0 (not perceivable) to 3 (strong), in steps of 0.5. The evaluation of the samples was carried out in duplicate and the results obtained by each panelist were averaged.

2.2. Results and discussion

2.2.1. Characterization of odor-active compounds in a Carmenere red wine.

Volatile compounds were isolated from Carmenere red wine by solvent extraction and SAFE distillation. The solvent extraction was performed at ambient temperature with addition of sodium chloride to avoid emulsions and facilitate phase separation. In the distillation process, the temperature was always kept below 40 °C to avoid compound deterioration and artifact formation. According to the sensory evaluation carried out by the trained panel, the distillate obtained showed the same fruity, cherry and spicy odor nuances, as the Carmenere red wine analyzed.

The volatiles isolated were separated into AV and NBV fractions (see Materials and Methods). AEDA application to both fractions revealed twenty-one odor active zones with FD factors ≥ 16 (Table 2.1). Some of the compounds with the highest FD factors were described as smelling fruity-sweet (**1, 3, 8, 13, 14, 19**), berry-like (**12, 16**), and spicy (**7, 10, 11**) (Figure 2.1), in agreement with the findings of the sensory analysis. Preliminary structural assignment of the odorants was carried out by comparing the retention indices and odor qualities with data of an in-house built database (Steinhaus,

Sinuco, Polster, Osorio, & Schieberle, 2008). To confirm the structural assignments, HRGC-O and HRGC-MS analysis were performed in parallel with the corresponding reference compounds.

The Carmenere red wine contained numerous volatile compounds in the NBV fraction, some of them present in high amounts. These might co-elute and interfere with odorants present at low concentration levels. To avoid this problem, a silica gel column fractionation was run to the NBV fraction. Elution was carried out with five different mixtures of pentane/diethyl ether, as described above. Individual fractions obtained were concentrated and analyzed by HRGC-O and HRGC-MS.

Besides the odorant compounds previously identified in the unfractionated NBV, six additional new odorant zones were localized. However, only three could be characterized as ethyl 2-hydroxypropanoate (sweet-like; RI 1371), hexan-1-ol (green grass; RI 1379) and ethyl decanoate (dried fruit; RI 1662). The other three odorant zones were described as smoky (RI 1487), phenolic (RI 1498) and woody-like (RI 1894), respectively. New odorant zones identified in the silica fractionation only reached the undiluted extract in AEDA analysis; therefore, no further effort was pursued to identify these uncharacterized odorant zones.

Table 2.1. Odor-active compounds ($FD \geq 16$) in the SAFE distillate obtained from Carmenere red wine.

no.	aroma compound ^a	odor quality ^b	RI ^c (FFAP)	FD ^d
1	2-/3-methyl-1-butanol	fruity, sweet	1202	≥ 1024
2	2-phenylethanol	flowery, rose	1918	≥ 1024
3	ethyl butanoate	fruity	1029	256
4	acetic acid	vinegar-like	1435	256
5	2-/3-methylbutanoic acid	cheese	1667	256
6	5-butyl-4-methyldihydro-2(3H)-furanone	coconut	1959	256
7	3-hydroxy-4,5-dimethyl-2(5H)-furanone	seasoning-like	2209	256
8	methyl 2-/3-methylbutanoate	fruity, sweet	1007	64
9	4-hydroxy-2,5-dimethyl-3(2H)-furanone	caramel, burnt sugar	2034	64
10	ethyl cinnamate	sweet, cinnamon	2132	64
11	4-allyl-2-methoxyphenol	clove-like	2159	64
12	ethyl 2-methylpropanoate	fruity, berries	947	32
13	ethyl 3-methylbutanoate	fruity, sweet	1066	32
14	ethyl hexanoate	fruity, sweet	1233	32
15	butanoic acid	cheese	1620	32
16	β -damascenone	plum, berries	1818	32
17	2-methoxyphenol	smoky	1863	32
18	benzyl alcohol	flowery	1877	32
19	ethyl 2-methylbutanoate	fruity	1045	16
20	3-(methylthio)propanal	cooked potato	1457	16
21	3-(methylthio)-1-propanol	cooked potato	1724	16

^a The compounds were identified by comparing their retention indices on capillary FFAP, mass spectra (MS-EI), and odor qualities as well as odor intensities as perceived during GC-O with the data obtained from reference compounds under the same conditions

^b Odor quality perceived at the sniffing port

^c Retention index

^d Flavor dilution factor determined by AEDA on capillary FFAP

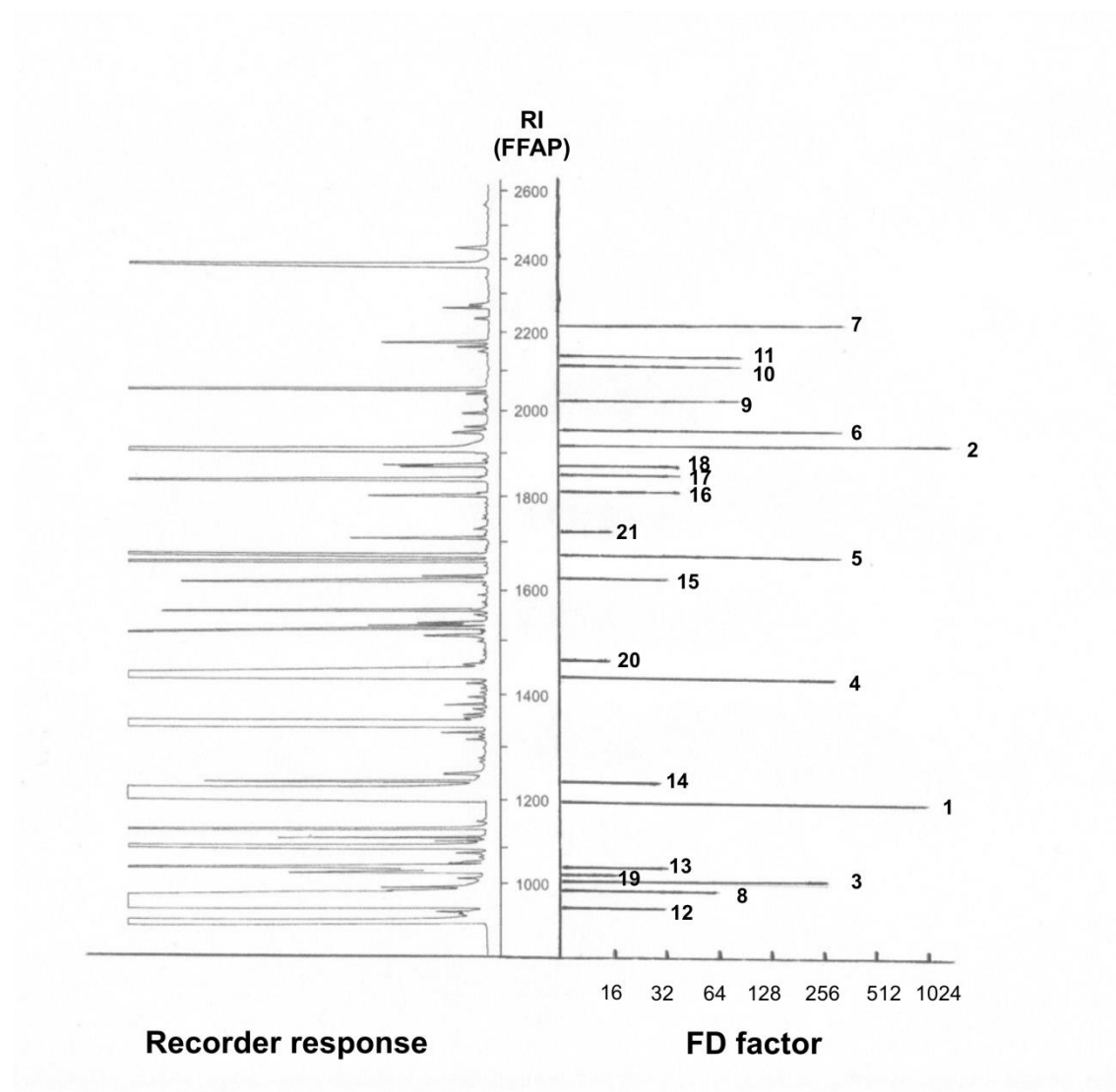


Figure 2.1. HRGC-FID chromatogram (left) and corresponding FD chromatogram (right) obtained by an AEDA applied to an unfractionated Carmenere red wine extract including the AV and NBV.

2.2.2. Aroma profile of Carmenere red wine

Major identified odor-active compounds agreed with those previously reported for other red wines. They are mainly synthesized by the yeast metabolism during the alcoholic fermentation (Ferreira et al., 2008; Frank et al., 2011). However, there was an odorant zone with a retention index of 1007 and dilution factor of 64 that caught our attention due to its fruity and sweet odor. By comparison of retention indices, mass spectra and odor qualities at the sniffing port with reference compounds this odorant zone was identified as a mixture of methyl 2-methylbutanoate (M2MB) and methyl 3-methylbutanoate (M3MB). Only few methyl esters compounds have been reported previously as wine aroma compounds in literature. Compounds such as methyl butanoate, methyl hexanoate, methyl octanoate and methyl decanoate were found at concentration values close to 1 µg/L in Bordeaux red wines (Antalick, Perello, & de Revel, 2010). Methyl esters could be formed in wine through the reaction between carboxylic acids and methanol. The concentration of methanol in wines varies between 30 and 35 mg/L and mainly results from enzymatic hydrolysis of methoxy groups in the pectin of berry cell walls during fermentation bution during the wine storage. It is important to consider that the sensory map developed by Casaubon et al., 2006 was built with Carmenere red wine samples without oak contribution, in order to study only the aroma contribution from the grape berries.

Additionally, the Carmenere red wine analyzed exhibit a market cooked apple-like aroma. This aroma descriptor is mainly attributed to the C₁₃-norisoprenoid compound β-damascenone. (Domínguez & Agosin, 2010) found in Carmenere red wines from Maipo and Colchagua valleys an OAV of 516 for β-damascenone. This OAV is an indicator that this compound is present well above its odor detection threshold and could be an important contributor of the Carmenere red wine aromas.

Aroma descriptors such as cooked potato, sweaty, butter-like and malty are related to compounds such as 3-(methylthio)propanal, 2-/3-methylbutanoic acid, 2,3-butandione and 2-methyl-1-butanol, respectively. All these compounds were described by Ferreira

et al., 2008 as constituents of the wine basal aroma. The aroma descriptor flowery that is one of the strongest attributes for all Carmenere samples analyzed could be related to the odorant compound 2-phenylethanol. However, as previously reported by Belancic and Agosin 2007a, Carmenere wines have a high aging potential, mainly related to their high content of C₁₃-norisoprenoids in their glycoside form. C₁₃-norisoprenoid compounds are related to flowery aroma nuances and could also be important contributors to Carmenere wine aroma.

Both, GC-O and aroma profile analysis did not reveal the presence of thiol compounds in Carmenere wine samples. Nevertheless, considering the previous work on Carmenere that reported the varietal thiols FFT, PhMT, 3SH and 3SHA (Domínguez & Agosín, 2010), the importance of volatile thiols will be reinvestigated in the present thesis by application of GC-O and AEDA experiments and an optimized extraction procedure and quantitation method for these compound in wine samples will be proposed.

Finally, Carmenere red wine also has a characteristic vegetable-like aroma, mainly attributable to methoxypyrazine compounds. However, the concentration of these compounds is closely dependent on vitivinicultural practices, such as sun exposure of the grape berries and terroir (Belancic & Agosin, 2007). It is worth to mention that, by GC-O, we located two odorant zones, the first with a RI = 1426 described as green and earthy and the other one with a RI = 1516 described as bell pepper. By comparison with referenced compounds, these were identified as 3-isopropyl-2-methoxypyrazine and 3-isobutyl-2-methoxypyrazine, respectively. However, both odorant zones only reached an FD of 2. Therefore, they have not been considered in further experiments.

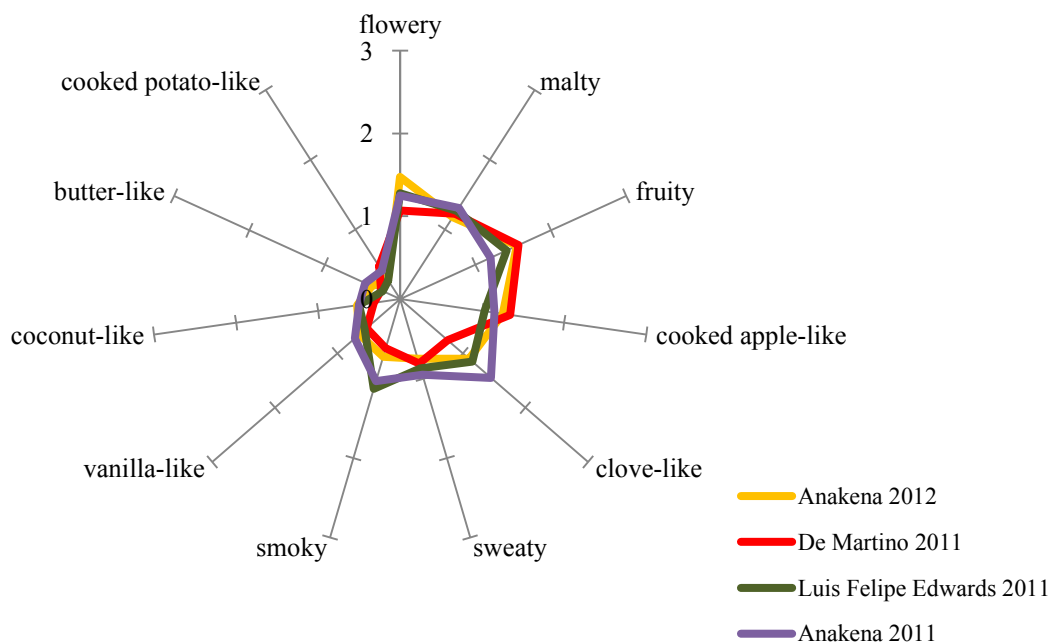


Figure 2.2. Aroma profiling of 4 different Carmenere red wines.

2.3. Conclusion.

By application of AEDA and HRGC-MS we identified 21 odor-active compounds with an $FD \geq 16$. Among these compounds, we identified two methyl esters M2MB and M3MB, not reported in red wines before. By descriptive aroma profiling, Carmenere red wine was characterized as fruity, flowery and spicy. Additionally, we found aroma descriptors such as smoky, coconut and vanilla-like. These aroma descriptors are related to phenols and lactones derived from oak wood during wine aging that give more complexity to the overall wine aroma.

3. Identification, quantitation and sensory evaluation of methyl 2- and methyl 3-methylbutanoate in varietal red wines

Carolina Pavez, Martin Steinhaus, Gerard Casaubon, Peter Schieberle, Eduardo Agosin

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Abstract

Background and Aims: Esters are the most important compound class contributing to fruity and berry-like aroma notes in wine. In this study, we aimed at identifying aroma-active compounds in Carmenere red wine with a focus on fruity smelling compounds.

Methods and Results: Application of an aroma extract dilution analysis to an aroma isolate obtained from Carmenere red wine by solvent-assisted flavour evaporation revealed, apart from well-known wine aroma compounds, two additional fruity smelling compounds, which were identified as methyl 2-methylbutanoate (M2MB) and methyl 3-methylbutanoate (M3MB). Quantitation of M2MB and M3MB in 16 different varietal red wines by stable isotope dilution assays resulted in concentrations clearly below (M2MB) and close or slightly above (M3MB) the respective odour threshold values. Spiking experiments based on a synthetic wine aroma model in combination with triangle tests did not result in significant sensory differences.

Conclusions: M2MB and M3MB do rather not contribute to the fruity aroma of red wines.

Significance of the Study: This is the first report on occurrence, concentration, and sensory impact of M2MB and M3MB in red wines.

Keywords: *ester, fruity aroma, red wine, aroma extract dilution analysis, sensory analysis*

3.1 Introduction

One of the most important attributes of wine quality is its aroma. In red wines, fruity aromas are mainly related to ethyl and acetate esters, like ethyl hexanoate, ethyl octanoate, ethyl 2- and ethyl 3-methylpropanoate, 3-methylbutyl acetate and hexyl acetate, among others. These esters are mostly produced by yeast cells through lipid and acetyl-CoA metabolism (Swiegers et al. 2005). They are often found in concentrations above their odor thresholds (Jackson 2008) and several belong to the so-called vinous aroma (Ferreira et al. 2008).

More complex fruity notes, such as blackberry, red berry or jam, commonly-cited sensory attributes for red wines, could not be fully explained by such compounds. For example, a new ethyl ester, ethyl 2-hydroxy-4-methylpentanoate, was recently reported in red wines and held responsible for a blackberry odor note). Several uncommon ethyl esters, such as ethyl 2-hydroxy-3-methylbutanoate, ethyl 2-hydroxy-4-methylpentanoate, and ethyl cyclohexanoate were recently identified in Port wines as important contributors to the specific fruity notes of this type of wines.

Pineau et al. (2009) recently reported that fruity aroma notes in red wines could result from the additive effect of certain esters, including those present below their threshold concentrations. Additionally, they showed that small variations in the concentration of esters could promote a significant change in the overall wine aroma.

In this study, part of a series aiming to characterize the odorant compounds of Carmenere red wines, we report the discovery of two methyl esters, their sensory thresholds and concentrations in 16 different commercial red wines.

3.2 Materials and methods

3.2.1 Wine samples

Aroma profiling was carried out in a 100% varietal Carmenere red wine of Anakena winery, 2011 vintage, produced at Las Cabras, Sixth Region, Chile. Fifteen commercial

red wines of several varieties and different countries were bought at a specialized wine store in Munich, Germany, and used for the quantitation studies.

3.2.2. Chemicals

The following reference odorants were obtained from Sigma-Aldrich Chemie, Taufkirchen, Germany: acetic acid, 4-allyl-2-methoxyphenol (eugenol), butanoic acid, 5-butyl-4-methyldihydro-2(3*H*)-furanone (whisky lactone), ethyl butanoate, ethyl cinnamate, ethyl hexanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 2-methylpropanoate, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolon) , 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 2-methoxyphenol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methylbutanoic acid, 3-methylbutanoic acid, methyl 2-methylbutanoate (M2MB), methyl 3-methylbutanoate (M3MB), 3-(methylthio)propanal and 3-(methylthio)-1-propanol. (*E*)- β -Damascenone was a gift from Symrise, Holzminden, Germany.

The labeled [$^2\text{H}_3$]-M2MB was prepared from [$^2\text{H}_4$]-methanol and 2-methylbutanoic acid, using an approach previously detailed for the synthesis of labeled ethyl butanoate.

3.2.3 Isolation of volatiles

To the wine (100 mL) sodium chloride was added until saturation. The mixture was extracted with diethyl ether (3×100 mL) and the combined organic phases were dried over anhydrous sodium sulfate. Non-volatiles were removed by solvent-assisted flavor evaporation (SAFE) at 40 °C (Engel et al. 1999). The SAFE distillate was separated into acidic (AV) and neutral/basic volatiles (NBV) by acid/base extraction as previously reported (Frank et al. 2011). The resulting fractions were concentrated (1 mL) using a Vigreux column (50×1 cm).

3.2.4 High resolution gas chromatography-olfactometry (HRGC-O) and high resolution gas chromatography-mass spectrometry (HRGC-MS).

HRGC-O was carried out with a 5160 Mega Series gas chromatograph (Carlo Erba Instruments; Milano, Italy), using FFAP and DB-5 fused silica capillaries (both $30 \text{ m} \times$

0.32 mm id, 0.25 μ m film thickness; J&W Scientific, Agilent Technologies, Waldbronn, Germany). Samples were introduced into the GC by the cold-on-column injection technique at 40 °C and using helium as carrier gas. The oven programs and the carrier gas flow rates were the same as described by Frank et al. (2011). At the end of the capillary column, the effluent was split 1:1 by volume using a Y-shaped glass splitter and two deactivated fused silica capillaries (50 cm $\times$ 0.20 mm id). One part was directed into an FID detector and the other part into a tailor-made sniffing port, kept at 220 °C. Calculations of linear retention indices (RI) were carried out after co-injection with a series of *n*-alkanes, as described previously (Van den Dool & Dec Kratz 1963).

HRGC-MS was performed using a HP 5890 gas chromatograph (Hewlett-Packard, Heilbronn, Germany) connected to a Finnigan type MAT 95 S mass spectrometer (Finnigan, Bremen, Germany) in the electron impact (EI) mode at 70 eV.

3.2.5 Quantitation of M2MB and M3MB by stable isotope dilution assay (SIDA) and high resolution gas chromatography-mass spectrometry (HRGC-MS).

Sodium chloride (10 g) and the internal standard, dissolved in diethyl ether, were added to the wine sample (100 mL) and the mixture was extracted with diethyl ether (2 $\times$ 50 mL). The organic phase was dried over sodium sulfate and submitted to SAFE distillation at 40 °C. The SAFE distillate was washed with aqueous sodium chloride (1 mol/L; 4 $\times$ 100 mL) and concentrated (200 μ L) by means of a Vigreux column (50 $\times$ 1 cm) and a microdistillation device (Frank et al. 2011).

HRGC-MS measurements were performed by means of two-dimensional heart-cut gas chromatography-mass spectrometry. The system consisted of a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland), a Trace Ultra GC (Thermo Scientific, Dreieich, Germany), a heated (250 °C) transfer line, a GC 3800 gas chromatograph (Varian, Darmstadt, Germany), and a Saturn 2200 ion trap mass spectrometer (Varian). The first GC (Trace) was equipped with a cold-on-column injector (Thermo), a column DB-FFAP (30 m $\times$ 0.32 mm i.d., 0.25 μ m film thickness; J&W, Agilent), a moving column stream switching system (MCSS; Thermo), as well as an FID (Thermo) and a sniffing port

(tailor-made) as monitor detectors. The temperature program for the first column was 40 °C for 2 minutes, followed by a gradient of 6 °C /min until 230 °C. The MCSS was connected via a heated (250 °C) transfer line consisting of an uncoated but deactivated fused silica capillary (0.32 mm i.d.) to a column DB-1701 (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W, Agilent) in the second GC (3800). Oven temperature program for this column was 40 °C for 2 minutes, followed by gradients of 8 °C /min until 130 °C and 40 °C /min until 240 °C. Volatiles conveyed by the MCSS time programmed to the second oven were refocused by a jet of cold nitrogen gas (-196 °C) applied to the end of the transfer line until the second oven was started. Mass chromatograms were recorded in the chemical ionization (CI) mode with methanol as reagent gas.

For the quantitation of M2MB and M3MB, calibration curves were prepared using five mixtures of defined amounts of analytes and labeled internal standard covering a concentration ratio range from 1:5 to 5:1. GC-MS measurements were carried out in duplicates ($RSD \leq 15\%$). Linear regression applied on the average values of the duplicates for the five calibration mixtures resulted in good linearity (M2MB: $R^2 = 0.9978$; M3MB: $R^2 = 0.9971$). Limit of detection (LOD) and limit of quantitation (LOQ) were approximated as the concentration at which the signal-to-noise ratio (S/N) was 3 (LOD) and 10 (LOQ). For both compounds, LOD and LOQ were 0.008 µg/L and 0.028 µg/L, respectively.

3.2.6. Odor thresholds

Odor thresholds of M2MB and M3MB were determined orthonasally in a synthetic wine matrix solution (11% ethanol, v/v; 5 g/L tartaric acid, pH adjusted to 3.4 with NaOH 1 mol/L), (ASTM 2005).

3.2.7. Sensory evaluation

Experiments on the sensory impact of M2MB and M3MB were based on a red wine aroma model consisting of the synthetic wine matrix solution detailed above and the 28 major red wine odorants (in their natural concentrations). A trained panel composed of

24 panelists performed the sensory evaluations (females, 35 – 48 years old, with 9 years' experience in wine sensory analyses). The panelists were recruited according to the ISO 8586-1 international standard (Sensory analysis – General guidance for the selection, training and monitoring of assessors) and accredited under international ISO quality standards (ISO 5495:2005) by the Chilean National Normalization Institute (INN). According to statistical analyses of variance with a confidence level of 95%, the panel was reproducible, repeatable and discriminate (data not shown).

Triangular tests according to ISO 4120:2004 were performed orthonasally to assess differences between a wine model solution with and without addition of both, M2MB and M3MB. Sensory tests were performed in a complete experimental model design, including the presentation of the samples to the panelists in all possible combinations.

Four triangular tests were run, according to the concentrations found in the 16 varietal red wines analyzed, as follows: **Test 1 (T1)**, M2MB 0.020 µg/L and M3MB 4.5 µg/L. **Test 2 (T2)**, M2MB 0.027, µg/L; and M3MB 5.4 µg/L. **Test 3 (T3)**, M2MB 0.020 µg/L. **Test 4 (T4)**, M3MB 5.4 µg/L.

Panelists evaluated the samples (20 mL; freshly prepared) in glass cups with cover. Data was analyzed using the sensory evaluation software Compusense Five 5.4 (Compusense, Inc., Ontario, Canada).

3.3 Results and discussion

3.3.1 Aroma-active compounds in a Carmenere red wine

The volatile fraction obtained from a Carmenere red wine after solvent extraction and SAFE distillation showed the same fruity, cherry-like, and spicy odor nuances as the original wine. The volatiles were separated into two fractions, one containing the acidic volatiles (AV), the other the neutral and basic volatiles (NBV). Application of an aroma extract dilution analysis (AEDA) (Schieberle and Grosch 1987) to AV and NBV fractions, respectively, revealed twenty-one aroma-active zones in the flavor dilution (FD) factor range of 16 to ≥ 1024 (data not shown).

Structural assignments of the odorants were carried out by comparing the retention indices and odour qualities with an in-house database available at the Deutsche Forschungsanstalt für Lebensmittelchemie. To confirm the structural assignments, the wine volatile fractions were then analyzed in parallel with the corresponding reference compounds by HRGC-O and HRGC-MS.

The odorants with the highest FD factor (≥ 1024) were identified as sweet, malty smelling 2-methyl- and 3-methyl-1-butanol, flowery smelling 2-phenylethanol, and fruity smelling ethyl hexanoate and ethyl 2-methylbutanoate. All these compounds are commonly found as aroma-active compounds in wine and are synthesized by yeast during fermentation. Further odorant zones with high FD factors have been previously reported as constituents of the vinous aroma, i.e. the wine odor matrix (Ferreira et al. 2000, 2008, López et al. 1999). Among these odorant zones the following aroma descriptors and structural assignments were found; fruity (ethyl 2-methylpropanoate, ethyl butanoate, and ethyl 3-methylbutanoate), berry-like ((*E*)- β -damascenone), seasoning-like (3-hydroxy-4,5-dimethyl-2(*5H*)-furanone), clove-like (4-allyl-2-methoxyphenol), and floral (ethyl cinnamate).

AEDA is generally accepted as an appropriate tool to define a set of potent aroma-active compounds that includes potential key aroma compounds. In this regard, an odorant zone with an FD factor of 64 and centered at a RI of 1007 caught our attention due to its particular fruity, sweet and cherry-like aroma. A thorough study by means of two-dimensional gas chromatography in combination with the comparison of parameters, such as odor quality, retention times, and mass spectra with respective data of reference compounds analyzed in parallel, allowed identifying M2MB (RI = 1006) and M3MB (RI = 1018) in this odorant zone (Figure 3.1). Even though M2MB has not been previously reported in wines, M3MB has been described as a volatile compound associated to the microbial flora in “Blaufränkisch” red grape berries. It is noteworthy that so far only few methyl esters have been found in wines. Antalick et al. (2010) reported methyl butanoate, methyl hexanoate, methyl octanoate, and methyl decanoate at concentrations close to 1 $\mu\text{g/L}$. Methyl esters could be formed in wine through the reaction between

carboxylic acids and methanol. The concentration of methanol in wines varies between 30 and 35 mg/L and mainly results from enzymatic hydrolysis of methoxy groups in the pectin of berry cell walls during fermentation (Ribéreau-Gayon and Glories, 2000).

3.3.2 Quantitation of M2MB and M3MB in varietal red wines

M2MB and M3MB were previously described as key odorants in strawberries and blueberries. However, the concentration - and sensory role - of these methyl esters in red wines have not been addressed so far. Therefore, we determined the concentrations of M2MB and M3MB in the Carmenere red wine analyzed previously and 15 additional commercial varietal red wines of different grapes and different origin. Quantitation was accomplished by stable isotope dilution assays using [$^2\text{H}_3$]-M2MB as isotopologue internal standard. Results (Table 3.1) showed concentrations of 0.020 $\mu\text{g/L}$ (M2MB) and 4.5 $\mu\text{g/L}$ (M3MB) in the Carmenere wine used for AEDA. In all 16 wine samples analyzed, concentrations for M2MB and M3MB ranged from 0.009 to 0.083 $\mu\text{g/L}$, and from 1.3 to 5.4 $\mu\text{g/L}$, respectively.

To get a first idea on the potential impact of M2MB and M3MB on the aroma of red wine, their odor detection thresholds in a model wine solution (11% v/v ethanol, pH 3.4) were determined. Results showed threshold values of 2.2 $\mu\text{g/L}$ for M2MB and 3.6 $\mu\text{g/L}$ for M3MB (Table 3.2). Thus, the concentration of M2MB was far below its threshold value in all of the 16 red wines analyzed, while M3MB showed concentrations close or slightly above its threshold. Therefore, M2MB would rather not contribute to the aroma of the red wines, whereas there could be an impact of M3MB. It is important to note that M2MB is a chiral compound. However, since the concentrations found in the entire wine samples were clearly well below the threshold of the racemate, no efforts were made to analyze the enantiomeric ratio.

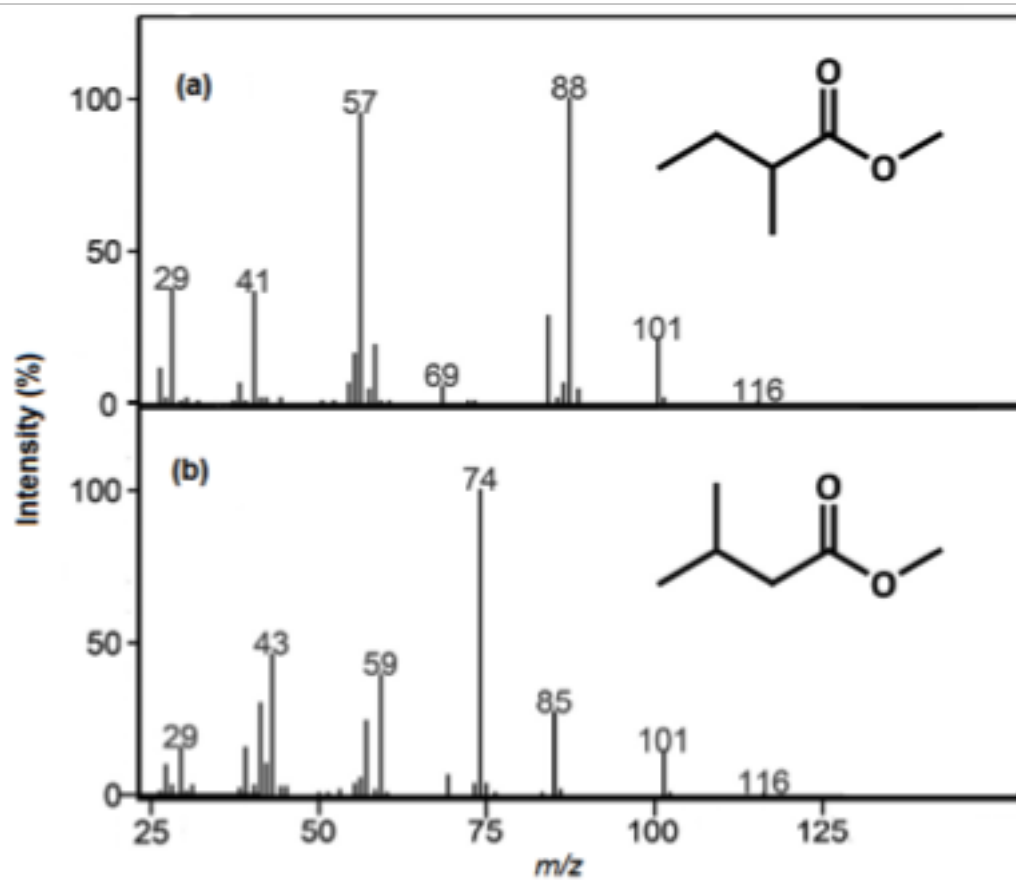


Figure 3.1. Mass spectra (EI, 70 eV) of M2MB (a) and M3MB (b).

Table 3.1. Concentrations and odor activity values of methyl 2-methylbutanoate and methyl 3-methylbutanoate in 16 different varietal red wines.

Nº	Grape variety	Harvest	Origin	Odor descriptors [†]	Conc. M2MB (µg/L)	Conc. M3MB (µg/L)	OAV [§] M2MB	OAV [§] M3MB
1	Carmenere‡	2011	Chile	Butter, cherry notes	0.020	4.5	0.01	1.3
2	Carmenere	2007	Chile	Pepper, a little bit pyrazine-like, slightly woody	0.056	1.4	0.03	0.4
3	Dornfelder	2009	Germany	Vegetable, sulphurous, cooked asparagus, raisins	0.083	5.2	0.04	1.5
4	Merlot	2011	France	Prunes, berries, red berry, berry jam, sweet	0.058	2.7	0.03	0.7
5	Carmenere	2011	Chile	Fruity, berries, like black berry jam, prunes, a little bit of butter	0.021	2.4	0.01	0.7
6	Carmenere	2010	Chile	Woody, berries, caramel, vanilla, pepper	0.032	1.9	0.01	0.5
7	Cabernet Sauvignon	2009	South Africa	Strong notes of 4-ethylphenol, leather, woody, alcohol very notorious	0.053	2.3	0.02	0.6
8	Cabernet Sauvignon	2011	France	Berry, cherry, citric, dried orange, a little bit of vanilla	0.027	5.4	0.01	1.5
9	Shiraz	2010	Australia	Red berry and cherry, pepper, wood and tobacco	0.047	1.4	0.02	0.4

Table 3.1. Concentrations and odor activity values of methyl 2-methylbutanoate and methyl 3-methylbutanoate in 16 different varietal red wines (Continued).

Nº	Grape variety	Harvest	Origin	Odor descriptors [†]	Conc. M2MB (µg/L)	Conc. M3MB (µg/L)	OAV [§] M2MB	OAV [§] M3MB
10	Malbec	2011	Argentina	Butter, berries (red berries), sweet, cherry jam, spicy, vanilla, woody	0.015	2.1	0.01	0.6
11	Nebbiolo	2010	Italy	Strawberry jam, sweet, banana, vanilla, spicy	0.040	2.2	0.02	0.6
12	Cabernet Sauvignon	2009	Chile	Butter, sweet, black berry, pepper, spicy, woody	0.073	3.3	0.03	0.9
13	Cabernet Sauvignon	2009	Italy	Strawberry, raspberry, sweet, spicy	0.046	1.3	0.02	0.4
14	Tempranillo	2011	Spain	Citric, like orange marmelade, woody	0.009	2.6	0.004	0.7
15	Pinot Noir	2008	New Zealand	Woody, cherries, like orange marmelade	0.043	3.2	0.02	0.9
16	Merlot	2009	Chile	Woody, plum, red berries	0.050	1.9	0.02	0.5

OAV, odor activity value; M2MB, methyl 2-methylbutanoate; M3MB, methyl 3-methylbutanoate.

[†] Odor descriptions given by the trained panel.

^{*} Carmenere red wine sample used for the screening experiments by AEDA analysis.

[§] Ratio of the concentration to the odor threshold in a model wine solution (cf. Table 2).

Table 3.2. Odor qualities and odor thresholds of methyl 2-methylbutanoate and methyl 3-methylbutanoate in water and in a model wine solution.

Odorant	Odor quality	Odor threshold (µg/L)	
		In water [†]	In model wine
M2MB	Fruity, apple-like	0.25	2.2
M3MB	Fruity	0.40	3.6

M2MB, methyl 2-methylbutanoate; M3MB, methyl 3-methylbutanoate.

[†]Schieberle and Hofmann, 1997.

3.3.3 Odor significance of M2MB and M3MB

Based on the data of Frank et al. (2011), a red wine aroma model consisting of a synthetic wine matrix solution (11% v/v ethanol, pH 3.4) and 28 major red wine odorants in their natural concentrations was spiked with different amounts of M2MB (racemic) and M3MB, covering the concentration ranges found in the analysis of the 16 different varietal red wines. Spiked samples were then orthonasally compared to the non-spiked red wine model in triangle tests. None of the tests resulted in a significant difference. Thus, M2MB and M3MB do rather not have an influence on the fruity aroma note in red wine, at least in the concentration ranges found in the 16 varietal red wines analyzed in this study. However, it has been shown that non-volatile components of wine could significantly influence the aroma release and volatility of odorant compounds and, therefore, influences the overall sensory perception (Ebeler and Thorngate 2009, Sáenz-Navajas et al. 2010). As this factor was not considered here, further experiments on the sensory impact of M2MB and M3MB might be conducted in a recombine including the non-volatile components of red wine.

3.4. Conclusion

AEDA and HRGC-MS analyses revealed the presence of M2MB and M3MB, two esters previously not reported in red wines. Quantitation of both methyl esters in 16 commercial varietal red wines resulted in concentrations clearly below the sensory threshold value for M2MB and close or slightly above the threshold for M3MB. Spiking experiments based on a synthetic red wine aroma model in combination with triangle tests, however, did not show a significant aroma impact of M2MB and M3MB at the concentration levels determined.

3.5. Acknowledgments

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4. Odorant screening and quantitation of thiols in Carmenere red wine by gas chromatography-olfactometry and stable isotope dilution assays.

Carolina Pavez, Martin Steinhaus, Peter Schieberle, Eduardo Agosin.

Abstract

Aroma extract dilution analysis was applied to a Carmenere red wine extract obtained from an affinity glass column chromatography with a mercurated agarose gel. The analysis revealed the presence of four odorant zones, identified as 2-furanylmethanethiol, 3-sulfanylhhexyl acetate, 3-sulfanyl-1-hexanol and 2-methyl-3-sulfanyl-1-butanol, described here for the first time in Carmenere red wines. Quantitation experiments by stable isotope dilution assays for all thiols identified were performed in the Carmenere red wine screened by AEDA and in other three Carmenere samples. Concentration values obtained were above the orthonasal odor detection threshold for all thiols. Triangular sensory experiments were performed in a red wine aroma model solution, with and without spiking thiols. Sensory experiments results showed significant sensory differences between the samples, suggesting that these compounds could influence the overall red wine aroma.

Keywords: *AEDA, red wine, GC-Olfactometry, thiols, SIDA.*

4.1. Introduction

The human nose detects some volatile thiols at very low odor thresholds (nanograms per liter), and these thiols are among the key aroma compounds of several foods, such as onion (Granvogl, Christlbauer, & Schieberle, 2004), grapefruit (Buettner & Schieberle, 1999), pink guava (Steinhaus, Sinuco, Polster, Osorio, & Schieberle, 2009), coffee (Czerny, Mayer, & Grosch, 1999), mango (Munafo, Didzbalis, Schnell, Schieberle, & Steinhaus, 2014) and wine. In wines, volatile thiols were first reported in white wine varieties (Darriet, Tominaga, Lavigne, Boidron, & Dubourdieu, 1995; Guth, 1997) and they are the main odorants responsible for the passion fruit, grapefruit, and box tree aromas in those wines. The volatile thiols most widely studied in wines are 3-sulfanyl-1-hexanol (3SH), 3-sulfanylhexyl acetate (3SHA), and 4-methyl-4-sulfanyl-2-pentanone (4MSP). They are present in grapes in an odorless form as they are linked to cysteine or glutathione. The adducts are cleaved by a yeast beta-lyase in the fermentation process (Fedrizzi et al., 2009; Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2012; Roland, Schneider, Razungles, et al., 2011). Other important volatile thiols in wines are 2-furanylmethanethiol (FFT) and phenylmethanethiol (PhMT). FFT is responsible for roasted coffee or toasty aromas and could be released by oak barrels during wine aging (Blanchard et al., 2001) PhMT is responsible for smoky aromas, and its origin in wines is unclear (Tominaga et al., 2003).

The contribution of thiols to the fruity aromas in white wines has been well addressed, especially in Sauvignon Blanc. (Guth, 1997; Tominaga, Furrer, et al., 1998) However, only a few studies on thiols have been performed in red wines. All studies examined Bordeaux red wines using gas chromatography-olfactometry, gas chromatography-mass spectrometry and an specific thiol work-up using mercury compounds (Tominaga, Murat, et al., 1998). The presence of 2-methyl-3-sulfanyl-1-propanol, 3SH, 3SHA, FFT, 4MSP, and 2-methyl-3-furanthiol was reported (Bouchilloux & Darriet, 1998; Kotseridis & Baumes, 2000; Tominaga, Blanchard, Darriet, & Dubourdieu, 2000). Additionally, aroma extract dilution analysis (AEDA) performed by Kotseridis and Baumes (2000) on Bordeaux red wines demonstrated that 3SH is an important odorant compound.

Recently, a correlation was found between sensory data and the concentration of thiol compounds (4MSP, 3SH, and 3SHA) in red wines from Languedoc, France. The concentration of 4MSP is well correlated with the blackcurrant aroma in the samples analyzed, while the compounds 3SH and 3SHA in high concentrations could act as enhancers of blackcurrant aroma perception.(Rigou, Triay, & Razungles, 2014)

Vitis Vinifera Carmenere is a red wine variety originating from the Bordeaux region, France. Carmenere was thought to be extinct after the phyloxera plague in Europe, but in 1994, it was rediscovered in Chile, where most of the vineyards of this grape variety are currently planted (Pszczółkowski, 2004). Sensory studies have characterized Carmenere red wine as vegetable-like, spicy, and fruity smelling. Vegetable-like aromas in this wine variety are mainly related to methoxypyrazine compounds (3-isobutyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine) (Belancic & Agosin, 2007), while the characteristics blackberry jam and spicy aromas could be related to norisoprenoids produced by hydrolysis from its glycoside precursors (Belancic & Agosin, 2007a).

In this work, we used gas chromatography-olfactometry (GC-O) to identify four odorant zones, corresponding to volatile thiols 3SH, 3SHA, FFT, and 2-methyl-3-sulfanyl-1-butanol (2M3SB) from a Carmenere red wine extract. The latter compound was identified and quantified here for the first time in Carmenere red wines. We also applied AEDA to calculate their dilution factors (FDs) and finally determined their concentrations and odor activity values (OAVs). Quantitation and calculation of OAVs was also done in three additional Carmenere red wine samples.

4.2. Materials and methods

4.2.1. Wine.

Four Carmenere red wine samples were used in this study. All samples were described in Table 4.1.

Table 4.1. Description of the Carmenere red wine samples used for the study.

Sample	Origin	Harvest	Sample description
CMR 1	Cachapoal valley, Chile	2011	complete fermentation without bottling process
CMR 2	Cachapoal valley, Chile	2011	commercial
CMR 3	Cachapoal valley, Chile	2012	complete fermentation without bottling process
CMR 4	Maipo valley, Chile	2011	commercial

4.2.2. Reference odorants and their labeled analogs.

FFT was purchased from Sigma-Aldrich, Taufkirchen, Germany and 3SH from Alfa Aesar, Karlsruhe, Germany. 3SHA was synthesized by reacting 3SH and acetyl chloride (Steinhaus et al., 2008). [$^2\text{H}_5$]-FFT, [$^2\text{H}_2$]-3SH, [$^2\text{H}_2$]-3SHA were synthesized according to a published procedures (Sen & Grosch, 1991; Steinhaus et al., 2009) 2M3SB and [$^2\text{H}_2$]-2M3SB were synthesized from 2-methyl-2-butenal, adapting the method reported Granvogl et al., 2004 for the synthesis of 2-methyl-3-sulfanyl-1-pentanol.

4.2.3. Chemicals and reagents.

The following chemicals were obtained from Sigma-Aldrich Chemie, Taufkirchen, Germany: acetyl chloride, lithium aluminum deuteride, lithium aluminum hydride, 2-methyl-2-butenal, piperidine, thioacetic acid, sodium chlorite, and sulfamic acid.

4.2.4. Isolation of volatile thiols from Carmenere red wine.

Sodium chloride was added to Carmenere red wine (100 mL) until saturation. The mixture was extracted with diethyl ether (200 + 100 + 100 mL), and the combined organic phases were dried over anhydrous sodium sulfate and concentrated (5 mL) by means of a Vigreux column (50 x 1 cm). The organic extract obtained was applied onto a mercurated agarose gel (1 g) in a glass column (0.5 cm i.d.). The column was rinsed with pentane/dichloromethane (2:1; 50 mL), and thiol compounds were eluted with dithiothreitol (10 mmol/L) in pentane/dichloromethane (2:1; 50 mL). The eluate was purified by solvent-assisted flavor evaporation (SAFE) at 40 °C.(Engel et al., 1999) The distillate obtained was concentrated (200 µL) using a Vigreux column and a microdistillation device.(Li, Schieberle, & Steinhaus, 2012)

4.2.5. High-resolution gas chromatography olfactometry (HRGC-O) and high-resolution gas chromatography mass spectrometry (HRGC-MS).

HRGC-O was performed with a 5160 Mega Series gas chromatograph (Carlo Erba Instruments; Milano, Italy) using FFAP and DB-5 fused silica capillaries (both 30 m × 0.32 mm i.d., 0.25 µm film thickness; J&W Scientific, Agilent Technologies, Waldbronn, Germany). Samples were introduced into the GC by the cold-on-column injection technique at 40 °C using helium as carrier gas. The oven program for both columns was 40 °C for 2 minutes, followed by a gradient of 6 °C/min until 230 °C. Helium flow was kept at 2 mL/min in the whole run. At the end of the capillary column, the effluent was split 1:1 by volume using a Y-shaped glass splitter and two deactivated fused silica capillaries (50 cm × 0.20 mm i.d.). One part was directed into an FID detector, and the other part into a tailor-made sniffing port kept at 220 °C. Calculations

of linear retention indices (RI) were carried out after co-injection with a series of *n*-alkanes, as described previously.(Dool, Den, & Kratz, 1963) HRGC-MS was carried out employing an HP 5890 gas chromatograph (Hewlett-Packard, Heilbronn, Germany) connected to a Finnigan type MAT 95 S mass spectrometer (Finnigan, Bremen, Germany) in electron impact (EI) mode at 70 eV.

4.2.6. Aroma extract dilution analysis (AEDA).

The dilution factors (FD) of the volatiles thiol compounds were determined by AEDA. For that purpose, the aroma isolate was stepwise diluted 1:10 using dichloromethane and the original extract, i.e. 1:10, 1:100 and 1:1000 dilutions. All dilutions were analyzed by HRGC-O until no odorants were detected by sniffing in the whole run. AEDA was performed by four sniffers and the results were averaged.(Li et al., 2012)

4.2.7. Quantitation of thiols by stable isotope dilution assays (SIDA).

HRGC-MS measurements were performed by means of two-dimensional heart-cut gas chromatography-mass spectrometry. The system consisted of a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland), a Trace Ultra GC (Thermo Scientific, Dreieich, Germany), a heated (250 °C) transfer line, a GC 3800 gas chromatograph (Varian, Darmstadt, Germany), and a Saturn 2200 ion trap mass spectrometer (Varian). The first GC (Trace) was equipped with a cold-on-column injector (Thermo), a column DB-FFAP (30 m × 0.32 mm i.d., 0.25 µm film thickness; J&W, Agilent), a moving column stream switching system (MCSS; Thermo), as well as an FID (Thermo) and a sniffing port (tailor-made) as monitor detectors.

The temperature program for the first column was 40 °C for 2 minutes, followed by a gradient of 6 °C/min until 230 °C. The MCSS was connected via a heated (250 °C) transfer line, consisting of an uncoated but deactivated fused silica capillary (0.32 mm i.d.) to a DB-1701 column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W, Agilent) in the second GC (3800). The oven temperature program for this column was 40 °C for 2 minutes, followed by gradients of 8 °C/min until 170 °C and 40 °C/min until 230 °C.

Volatiles that were conveyed to the second oven by time control of the MCSS were refocused by a jet of cold nitrogen gas (-196 °C) applied to the end of the transfer line until the second oven was started. Mass chromatograms were recorded in the chemical ionization (CI) mode with methanol as reagent gas.

The concentrations of the stock solutions of the labeled internal standards [$^2\text{H}_5$]-FFT, [$^2\text{H}_2$]-2M3SB, [$^2\text{H}_2$]-3SH, and [$^2\text{H}_2$]-3SHA were determined from GC-FID analyses using methyl octanoate as an internal standard and detector signal correction by application of response factors as described previously (Sen, Laskawy, Schieberle, & Grosch, 1991). For the quantitation of the thiols in wine, a calibration curve was prepared for each compound of defined amounts of analytes and labelled internal standard that covered a concentration ratio ranging from 1:5 to 5:1. The limit of detection (LOD) and limit of quantitation (LOQ) were approximated as the concentration at which the signal-to-noise ratios (S/N) were 3 and 10, respectively.

4.2.8. Odor threshold determination.

The odor threshold of 2M3SB was determined orthonasally in a synthetic wine matrix solution (11% ethanol, v/v; 5 g/L tartaric acid, pH adjusted to 3.4 with NaOH 1 mol/L). This was done following the ASTM E679-04 procedure for the determination of odor and taste thresholds by a forced-choice ascending concentration series method of limits. For the compounds FFT, 3SH, and 3SHA, the odor threshold values were those reported previously in the literature for a synthetic wine matrix solution. (Roland, Schneider, Razungles, et al., 2011; Tominaga et al., 2000)

4.2.9. Sensory experiments.

Experiments on the sensory impact of the thiols, FFT, 2M3SB, 3SH, and 3SHA were based on a red wine aroma model consisting of the synthetic wine matrix solution described above and the 28 major red wine odorants in their natural concentrations (Frank et al., 2011). Triangular tests, according to ISO 4120:2004, were performed to assess differences between a wine model solution with and without the addition of the

four thiols identified in the Carmenere red wine samples. Five triangular tests were run with concentrations of thiol compounds over their odor detection threshold in a synthetic wine matrix solution as follows: **Test 1 (T1)**, FFT 4 ng/L; **Test 2 (T2)**, 2M3SB 40 ng/L; **Test 3 (T3)**, 3SH 100 ng/L; **Test 4 (T4)**, 3SHA 5 ng/L; **Test 5 (T5)**, FFT 4 ng/L, 2M3SB 40 ng/L, 3SH 100 ng/L, and 3SHA 5 ng/L. The solutions were presented in Teflon vessels with a cover and coded with three-digit numbers. The panel consisted of 18 panelists trained in sensory analysis and were recruited from the German Research Centre for Food Chemistry.

4.3. Results and discussion

4.3.1. Volatile thiol screening.

HRGC-O performed with the Carmenere red wine extract using FFAP and DB-5 capillary columns revealed four odorant zones that could be associated with thiols. By comparison of the retention indices, odor quality and mass spectra with reference compounds, the respective odorants were identified as FFT, 2M3SB, 3SH, and 3SHA (Table 4.2). AEDA of a Carmenere extract particularly revealed 3SH, with an FD factor of 100, as a major aroma active compound. This was in agreement with results previously reported for other red wine varieties (Cabernet Sauvignon and Merlot) from Bordeaux, France. The presence of 3SH was found in these wine samples with an FD of 625.(Kotseridis & Baumes, 2000)

Table 4.2 Thiols identified in a Carmenere red wine extract analyzed by GC-olfactometry.

thiol compound ^a	odor quality ^b	LRI ^c		FD ^d
		FFAP	DB-5	
FFT	roasted coffee	1430	907	1
3SHA	box-tree	1716	1244	10
2M3SB	sweet onion	1726	1016	10
3SH	grapefruit	1839	1121	100

^a The compound was identified by comparing its mass spectra (MS-EI), retention indices on capillary columns FFAP and DB-5 as well as its odor quality perceived by GC-O with data obtained from reference compounds.

^b Odor quality perceived at sniffing port.

^c Linear retention index.

^d Flavor dilution factor as determined by AEDA.

4.3.2. Quantitation of volatile thiols in Carmenere red wine.

Volatile thiols have been widely studied in white wines, especially in Sauvignon Blanc. The concentration range of volatile thiols such as 4MSP, 3SH, and 3SHA has been well described for this type of wine (Coetzee & du Toit, 2012). On the other hand, there is no information about the concentration range for red wines, especially for the Carmenere variety. Therefore, we performed the quantitation of the thiol compounds identified above in four Carmenere red wine samples.

Calibration parameters (Table 4.3) showed that the analytical method used for quantitation experiments was linear in the range of calibration. Additionally, all measurements were made in duplicate, resulting in a RSD $\leq$ 15%. Quantitation showed concentrations of 33.1 ng/L (FFT), 530 ng/L (2M3SB), 617 ng/L (3SH) and 10.5 ng/L (3SHA) in the Carmenere red wine used for AEDA (Table 4.4). In the other 3 samples

analyzed, the concentration ranges of thiols were as follows: 0.0200 – 38.7 ng/L (FFT), 0.110 – 306 ng/L (2M3SB), 422 – 774 ng/L (3SH) and 8.20– 22.2 ng/L (3SHA).

It is noteworthy to note that the compound 2M3SB (Figure 4.1) has previously only been reported in only Sauternes wines in a concentration range of 118 to 185 ng/L.(Sarrazin et al., 2007) Sauternes wines are traditional AOC French wines made from Sauvignon Blanc, Semillon, or Muscatel grapes contaminated by noble rot (*Botrytis cinerea*). (Bailly, Jerkovic, Marchand-Brynaert, & Collin, 2006) Importantly, Carmenere grapes have a late harvest in order to prevent a high concentration of methoxypyrazines, which produces strong and undesirable vegetable-like aroma note. Overripe grapes are also used in the winemaking process for Carmenere red wine to produce wines with marked fruity aromas. However, this oenological practice could promote the occurrence of botrytis if it is not conducted carefully.(Pszczółkowski, 2004) Therefore, the compound 2M3SB could be a good marker for botrytized red – or white - wines.

Additionally, the OAV was determined for each thiol compound quantitated in the Carmenere red wines. This value is the ratio between the concentration of the thiol compound in the wine sample and its odor detection threshold in a synthetic wine solution. The OAVs (Table 4.5) for thiol compounds in all samples analyzed showed that the concentrations of these compounds are clearly above their odor detection threshold and therefore could influence the Carmenere red wine aroma.

Table 4.3. Calibration parameters of the quantitation method.

compound	R ²	slope	LOD ^a (ng/L)	LOQ ^b (ng/L)
FFT	0.9964	0.9690	0.1500	1.350
3SHA	0.9990	0.9844	0.02000	0.1700
2M3SB	0.9995	0.6798	0.1100	1.030
3SH	0.9989	0.8531	0.1500	1.360

^a Limit of detection.^b Limit of quantitation.

4.3.3. Sensory experiments.

Triangular tests were performed to evaluate whether thiol compounds in concentrations above their odor threshold were able to change the overall aroma in a red wine model solution containing the 28 most important red wine odorants. The results for all five tests showed that the additions of thiol compounds could be differentiated by the panelists in all triangular tests, with a confidence level of 95% and a p value of 0.05.

Red wine is a very complex matrix and the aroma perception is also influenced by the non-volatile part, such as phenolic compounds (Sáenz-Navajas et al., 2010). Regarding the relationship between thiols and the non-volatile part of red wine, it has been reported that due to the nucleophilic properties of these compounds, they could experience addition reactions with phenolic compounds (Blanchard, Darriet, & Dubourdieu, 2004; Cheynier & Trousdale, 1986). This phenomenon could influence the volatility of thiol compounds and reduce their sensory impact.

Finally, 2M3SB is a compound with two chiral centers that implies the existence of four different odorants. We determined the orthonasal odor threshold for the racemic mixture of 2M3SB and obtained a very low value of 0.2 ng/L in a synthetic wine matrix. Chiral

compounds with diastereoisomeric conformers that could adopt *like* and *unlike* configuration pairs have been previously described. *Like* and *unlike* are a terminology to describe the relative configuration of a diastereoisomer. A molecule has a *like* configuration if the descriptor pairs are “RR” or “SS”, whilst for *unlike* configuration descriptor pairs are “RS”. *Unlike* configuration of 2M3SB exhibit a strong onion-like note with a low odor threshold of 4 pg/L in air. This configuration could enhance meaty and boiled meaty aromas in food matrices in a concentration range of 0.1 to 1 µg/L and impart cooked vegetable-like and meaty notes at concentrations from 1 to 100 µg/L. On the other hand, *like* configuration of 2M3SB exhibit herbaceous, onion-like and leek-like aromas with odor threshold of 400 pg/L. Additionally, this configuration of 2M3SB could enhance the fruity character of exotic fruits at concentrations above 10 µg/L (Acuña, Gautschi, & Kumli, 2003) Therefore, further experiments should be conducted in order to determine the enantiomeric ratio of 2M3SB in Carmenere red wine samples to elucidate its contribution to the overall aroma in this wine variety.

Table 4.4. Concentration of thiols in 4 different Carmenere red wine samples.

sample	FFT (ng/L)	2M3SB (ng/L)	3SHA (ng/L)	3SH (ng/L)
CMR 1	33.1	530.2	10	617.4
CMR 2	nd ^a	306.2	8	422.2
CMR 3	31.9	35.2	21	774.5
CMR 4	38.7	< 1.03 ^b	22	759.9

^a Below of detection limit for FFT (0.15 ng/L).

^b Below of quantitation limit for 2M3SB (1.03 ng/L).

Table 4.5. Odor thresholds and odor activity values for the thiols quantitated in the Carmenere red wines.

wine sample	FFT		2M3SB		3SHA		3SH	
	OT (ng/L) ^a	OAV ^b	OT (ng/L) ^c	OAV ^b	OT (ng/L) ^d	OAV ^b	OT (ng/L) ^c	OAV ^b
CMR 1	0.4	83	0.2	2651	4.2	2	60	10
CMR 2	0.4	-	0.2	1531	4.2	2	60	7
CMR 3	0.4	80	0.2	176	4.2	5	60	13
CMR 4	0.4	97	0.2	-	4.2	5	60	13

^a Odor threshold in synthetic wine solution according to Blanchard et al., 2001.

^b Odor activity values calculated from the concentrations determined in the quantitation experiments (Table 3).

^c Odor threshold in synthetic wine solution determined as described above.

^d Odor threshold in synthetic wine solution according to Roland et al., 2011.

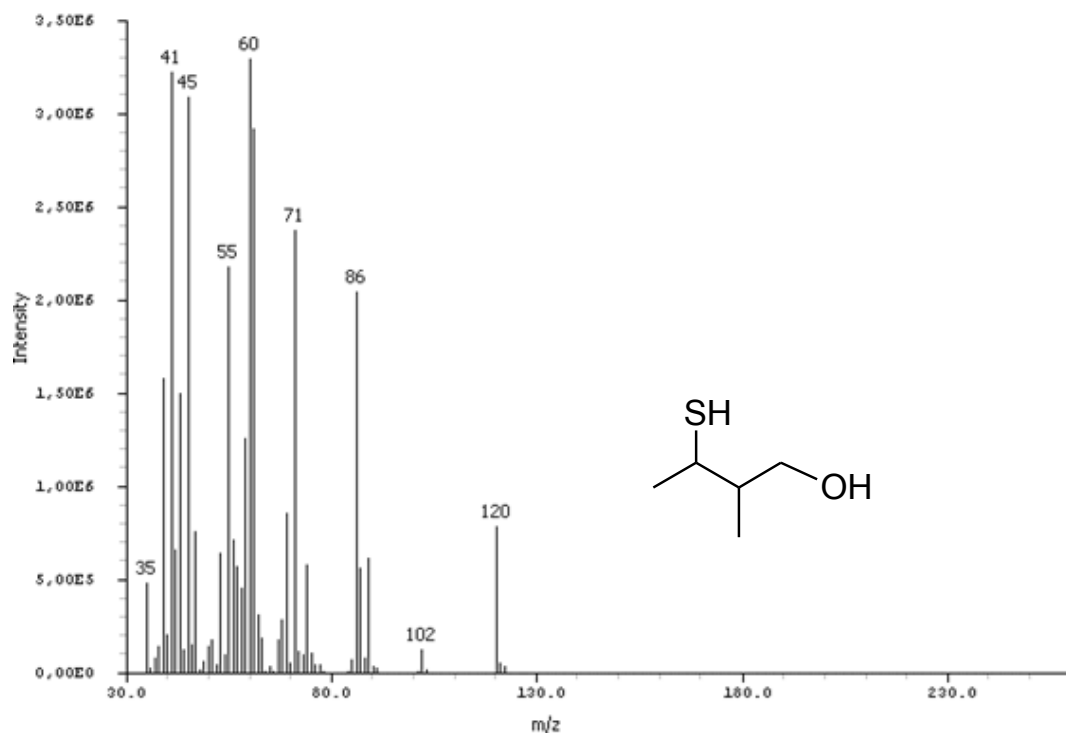


Figure 4.1. Mass spectrum and chemical structure of 2M3SB.

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5. Simultaneous quantitation of five important aroma-active thiols in Carmenere red wine samples by an optimized extraction procedure

Carolina Pavez, Chloé Capitaine, Eduardo Agosin, María Inés Espinoza

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Abstract

An optimized and novel derivatization reaction with pentafluorobenzylbromide performed in an aqueous reconstituted thiol solution from a pentane wine extract, followed by solid-phase micro-extraction (SPME), was developed by mean of GC-NCI-MS for the quantitative analysis of the odor-generating volatile thiols in wine, 3-mercapto-1-hexanol (3MH), 3-mercaptohexyl acetate (3MHA), 4-mercapto-4-methyl-2-pentanone (4MMP), phenylmethanethiol (PhMT), and 2-furanylmethanethiol (FFT) in a single chromatographic run. Validation experiments revealed recovery values ranging from 94% to 119% for red wines and from 65% to 111% for white wines. Repeatability and reproducibility values for whole volatile thiols, expressed in RSD, were below 15%. The determined detection limits were 0.76 ng/L for 3MH, 1.10 ng/L for 3MHA, 0.17 ng/L for 4MMP, 0.25 ng/L for PhMT, and 0.06 ng/L for FFT. Detection limits for all thiols were below the odor detection threshold determined in a wine model solution; the latter is indicative that the optimized method proposed is suitable for volatile thiol analysis in wine samples.

Keywords: *Gas Chromatography; Solid Phase Micro Extration; Wine Aromas; Volatile Thiols.*

5.1. Introduction

The human nose detects some volatile thiols at very low odor thresholds (nanograms per liter), and these thiols are among the key aroma compounds of several foods, such as onion (Granvogl et al., 2004), grapefruit (Buettner & Schieberle, 1999), pink guava (Steinhaus et al., 2009), coffee (Czerny et al., 1999), mango (Munafo et al., 2014), and wine.

In wines, volatile thiols were first reported in white wine varieties (Darriet et al., 1995; Guth, 1997) and they are the main chemicals responsible for the passion fruit, grapefruit, and box-tree aromas in those wines. The most widely studied volatile thiols in wines are 3-sulfanyl-1-hexanol (3SH), 3-sulfanylhexyl acetate (3SHA), and 4-methyl-4-sulfanyl-2-pentanone (4MSP). They are present in grapes in an odorless form, linked to cysteine or glutathione, and in the fermentation process the adducts are cleaved by the yeast via beta-lyase (Fedrizzi et al., 2009; Peña-Gallego et al., 2012; Roland, Schneider, Razungles, et al., 2011). Other important volatile thiols in wines are 2-furanylmethanethiol (FFT) and phenylmethanethiol (PhMT). FFT is responsible for roasted coffee or toasty aromas and could be released by oak barrels during alcoholic fermentation (Blanchard et al., 2001). PhMT is responsible for smoky aromas and its origin in wines is unclear (Tominaga et al., 2003).

The chromatographic analytical determination of thiol compounds at the concentrations present in wine is quite difficult, particularly because of the poor sensitivity for the tailing peaks, due to the interaction of thiol functionality with the stationary phases (Mateo-Vivaracho, Ferreira, & Cacho, 2006). Additionally, thiol compounds can easily react with oxygen and other oxidants (Hofmann, Schieberle, & Grosch, 1996) and may form complexes and precipitates with different metal ions (Nikolantonaki & Waterhouse, 2012).

First, volatile thiol analysis was performed using the property of thiols to form complexes with organomercury compounds. Tominaga et al. (1998) proposed the dichloromethane extraction of thiols from the wine matrix, followed by the reversible combination with *p*-hydroxymercurybenzoate in an anion exchange column. Finally,

volatile thiols are released with cysteine (Tominaga, Murat, et al., 1998). Another strategy is to form a mercury complex by means of a mercurated agarose gel in a glass column with subsequent elution with dithiothreitol in dichloromethane/pentane and (Full & Schreier, 1994). These strategies make it possible to isolate thiol compounds from the matrix, but they are very time consuming, use a large quantity of sample and organic solvents and mercury is extremely toxic for the humans.

In order to improve the sensitivity of volatile thiol analysis, some strategies have been proposed, based on the derivatization of the thiol function (Guth, Hofmann, Schieberle, & Grosch, 1995; Mateo-Vivaracho et al., 2006). Guth et al. (1995) used 4-vinylpyridine as a derivatizing reagent to enhance thiol stability. The use of 2,3,4,5,6-pentafluorobenzyl bromide (PFBBBr) was proposed, specifically for wine analysis, as a derivatizing reagent for thiol compounds (Capone, Sefton, & Jeffery, 2011; Dagan, Reillon, Roland, & Schneider, 2014; Mateo-Vivaracho, Cacho, & Ferreira, 2007; Mateo-Vivaracho et al., 2006; Mateo-Vivaracho, Zapata, Cacho, & Ferreira, 2010). The derivatization reaction with PFBBBr, due to the electronegative nature of this compound, gives the opportunity to perform the analysis by using an electron capture detector (ECD) or negative chemical ionization (NCI) mass spectrometry, making the measurements very sensitive and selective (Mateo-Vivaracho, Cacho, & Ferreira, 2008). PFBBBr derivatives for wine analysis in combination with solid phase extraction (SPE) for isolation of volatile thiols from the wine matrix have been exhaustively investigated (Mateo-Vivaracho et al., 2007; Mateo-Vivaracho, Cacho, & Ferreira, 2008; Mateo-Vivaracho et al., 2010). However, despite all the improvements made to the method, authors still have reported matrix effects and cross-contamination problems, resulting in poor linearity and repeatability of the method, especially for the determination and quantitation of 3SH (Mateo-Vivaracho et al., 2010).

Additionally, other strategies that involve PFBBBr derivatives have been reported: an on-fibre derivatization reaction using a solid phase microextraction (SPME) fibre (Mateo-Vivaracho et al., 2006), liquid-liquid extraction followed by derivatization and SPME extraction of the derivatives from the extract (Capone et al., 2011), prior on-cartridge

SPE derivatization and further SPME extraction of the derivatives from the SPE eluates (Rodríguez-Bencomo, Schneider, Lepoutre, & Rigou, 2009) and direct derivatization from the wine followed by SPME extraction (Dagan et al., 2014). All these methodologies make the analysis easier, repeatable, and reproducible, but they are developed only for the measurement of a specific thiol compound or, at maximum, three thiol compounds together.

Recently, was proposed ethyl propiolate as a new derivatizing agent to quantitate 4MSP, 3SH, and 3SHA in wines (Herbst-Johnstone, Piano, Duhamel, Barker, & Fedrizzi, 2013). However, the detection limit of the analytical method proposed for 4MSP is above its odor detection threshold in wines. Additionally, the pH value required for derivatization could promote the hydrolysis of 3SHA.

The aim of this work was to develop an analytical method to identify and quantitate the most important volatile thiols present in wines in a single chromatographic run. For this purpose, an optimized and novel derivatization reaction with PFBBBr was carried out in an aqueous redissolved thiols solution from a pentane wine extract previously evaporated to dryness, followed by solid-phase microextraction (SPME) to avoid matrix problems and enhance the sensitivity of the method.

5.2. Materials and methods

5.2.1. Reagents and chemicals.

The following reagents and chemicals were obtained from the suppliers given in parentheses: *n*-Pentane, reagent grade (JT Baker, Phillipsburg, USA); anhydrous sodium sulphate, ethanol, HPLC grade (Lichrosolv); ethylenediaminetetraacetic acid (EDTA) sodium chloride and tartaric acid (Merck, Darmstadt, Germany); 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), L-cysteine hydrochloride, methoxyhydroxylamine hydrochloride 98%, and PFBBBr (Sigma-Aldrich, Schnellendorf, Germany), divinylbenzene/polydimethylsiloxane (DVB/PDMS) SPME fiber (Supelco, Bellefonte, PA, USA).

5.2.2. Reference odorants.

3SH and 4MSP were from Chemos GmbH (Regenstauf, Germany). PhMT and FFT and the internal standard 2-phenylethanethiol (2PhEtSh) were from Sigma-Aldrich. Labelled [$^2\text{H}_{10}$]-4-methyl-4-sulfanyl-2-pentanone (d_{10} -4MSP) was from Nyseos (Montpellier, France) and [^2H]-3-sulfanyl-1-hexanol (d -3SH) was gift from Dr. Alvaro Cañete and synthesized in the Chemistry Faculty, Pontificia Universidad Católica de Chile according to the protocol described elsewhere (Hebditch, Nicolau, & Brimble, 2007).

5.2.3. Syntheses.

The thiol 3-sulfanylhhexyl acetate was synthesized by reacting 3-sulfanyl-1-hexanol and acetyl chloride in dichloromethane, as previously reported (Steinhaus et al., 2008).

5.2.4. The model wine preparation and wine samples.

The model wine solution consisted of a 10% v/v ethanol solution at pH 3.5, adjusted with tartaric acid. Wine samples were purchased from a local wine shop (Santiago, Chile).

5.2.5. Proposed method.

A wine aliquot (20 mL), EDTA (5 g/L; 0.2 mL), and L-cysteine (0.1 M; 0.6 mL) were mixed in a screw-capped glass vial. The mixture was shaken with a vortex for 2 min. Then, the internal standards 2PhEtSh (350 $\mu\text{g/L}$; 15 μL), d_{10} -4MSP (606 $\mu\text{g/L}$; 15 μL), and d -3SH (2016 $\mu\text{g/L}$; 15 μL) were added and the vials were shaken for another 2 min. Finally, methoxyhydroxylamine hydrochloride (0.2 g) was added. The glass vials were gently purged with nitrogen, sealed, and incubated at 55 °C for 45 min.

After the incubation period, samples were cooled to room temperature transferred to a funnel and pentane was added (3×20 mL). The solvent extraction was performed with the addition of sodium chloride to avoid emulsions and facilitate phase separation.

Organic phases were collected, dried over anhydrous sodium sulfate, and transferred into a headspace vial and evaporated to dryness.

Volatile thiols were redissolved in distilled water (5 mL), followed by addition of an aqueous solution of DBU (5% v/v; 1 mL) and PFBBBr solution (20 μ L PFBBBr in 50 mL ethanol; 0.1 mL). The derivatization reaction was performed by stirring the solution at room temperature for 20 min. Derivatized thiols were extracted by a DVB/PDMS SPME fiber at 70 °C for 45 min, with addition of NaCl (2 g) and tartaric acid (0.5 g). Finally, thiols were desorbed into a gas chromatograph (GC) inlet at 250 °C for 20 min.

5.2.6. GC-NCI-MS analysis.

Chromatographic analysis was performed using a GC Shimadzu QP-2010 Ultra coupled to a quadrupole mass spectrometer detector. The temperature of the inlet port was kept at 250 °C during the whole run in splitless mode. The capillary column was a HP1-MS (30 m $\times$ 0.25 mm id, 0.25 μ m film thickness) (J&W Scientific, Agilent Technologies, Santa Clara, CA, USA). The temperature program of the oven started at 50 °C for 2 min, temperature was raised up to 180 °C at 6 °C /min, then to 195 °C at 2 °C /min, and finally to 300 °C at 100 °C /min, where it remained for 5 min. The ion source was operated in NCI mode using methane as the reagent gas (pressure 300 KPa). The temperature of the ion source was kept at 220 °C and the interface at 270 °C. Detection was performed in single ion monitoring (SIM) mode. Quantitation and qualification ions, retention times of volatile thiols analyzed, and internal standards are listed in Table 5.1.

5.2.7. Method validation.

The analytical method proposed was evaluated by the following parameters: linearity, accuracy (recovery), repeatability, reproducibility, limits of detection (LOD), and quantification (LOQ).

Linearity was evaluated by a calibration curve in a wine model solution with five interval-spaced levels for each compound analyzed, as follows: from 0.1 to 9.2 ng for FFT; from 0.1 to 7.1 ng for PhMT; from 0.2 to 8.0 ng for 4MSP; from 0.2 to 16.0 ng for

3SHA; and from 2.1 to 68.3 ng for 3SH. To develop the calibration curves, three different internal standards were used: d-3SH at 30.2 ng to quantify 3SH and 3SHA; d₁₀-4MSP at 9.1 ng to quantify 4MSP; and 2PhEtSh at 5.3 ng to quantify FFT and PhMT. All calibration points were analyzed in triplicate.

Accuracy was expressed as recovery percentage and evaluated by spiked commercial red and white wine with a defined quantity of the volatile thiols studied. In order to evaluate the repeatability and reproducibility of the method, these wine samples were measured in triplicate on different days by different analysts. Repeatability and reproducibility results were expressed as the mean of residual standard deviation (RSD %).

LODs and LOQs were calculated, in triplicate, as the concentration at which the signal-to-noise ratios (S/N) were 3 and 10, respectively.

5.2.8. Sensory analysis of Carmenere red wine samples.

In order to obtain a fruity aroma profile and elucidate if thiols could influence fruity aromas of the Carmenere red wines analyzed. All the 13 Carmenere samples were submitted to sensory analysis using a trained panel. Panelists were recruited from Centro de Aromas y Sabores, DICTUC and were composed by 8 females with a large experience in sensory analysis of wine samples. Before sample analysis all panelists were calibrated to recognize the different fruity attributes from aromatic standards. Training consisted of a blind evaluation of aromatic standards prepared with a maceration of fruits or fruit liquor (e.g. cassis liquor). After recognition training, panelists developed a vocabulary and were trained in aroma intensity at different concentration levels for each attribute. Presentation of the samples was balanced, performed in duplicate and evaluated in two sessions (7 samples in the first session and 6 samples in the second session).

Table 5.1. Retention times, quantifier and qualifier ions of the pentafluorobenzyl-thiols derivatives and the corresponding internal standards analyzed by means of GC-NCI-MS with methane as reagent gas.

Compound	Type	Retention time (min)	Quantifier ion (m/z)	Qualifier ion (m/z)
FFT	Target	20.38	274	113
PhMT	Target	23.62	284	162
2PhEtSh	Internal standard	26.07	135	194 - 171
3SH	Target	23.83	133	194 - 213
3SHA	Target	25.71	175	59 – 113 - 194
d-3SH	Internal standard	23.80	134	194 - 213
4MSP (Z) and (E)	Target	23.83 – 24.02 ^a	160	194 – 301
d ₁₀ -4MSP (Z) and (E)	Internal standard	23.70 – 23.88 ^a	170	194 - 301

^a Retention times of the (Z) and (E) 4MMP-methoximes.

5.3. Results and discussion

5.3.1. Protective methoximation reaction of 4MSP.

Measurement of 4MSP is quite difficult, due to its fragmentation in mass spectrometry; 4MSP presents a weak abundance of high mass fragments in its mass spectrum, either in electron impact ionization or chemical ionization (Dagan et al., 2014). On the other hand, 4MSP has the lowest yield of derivatized product in the reaction with PFBBR. As reported by Mateo-Vivaracho et al. (2008), the derivatization reaction is affected by the ability of 4MSP to form an intramolecular hydrogen bond between the carbonyl functional group and the thiol group in the beta position, leaving the thiol group unavailable to react with PFBBR.

According to our experimental data, 4MSP exhibits no formation of the derivative product without a methoximation reaction. Therefore, methoximation is a crucial step to quantitatively evaluate 4MSP in wine samples. Additionally, and according to previous reports (Mateo-Vivaracho et al., 2008), the methoximation reaction produces two chromatographic peaks in NCI ionization mode, either 4MSP or d_{10} -4MSP at m/z 160 and m/z 170, respectively. These two chromatographic peaks correspond to the formation of (Z)-methoxime and (E)-methoxime (Figure 5.1). Calibration curve and quantitation experiments were performed with the sum of both signals, for all samples.

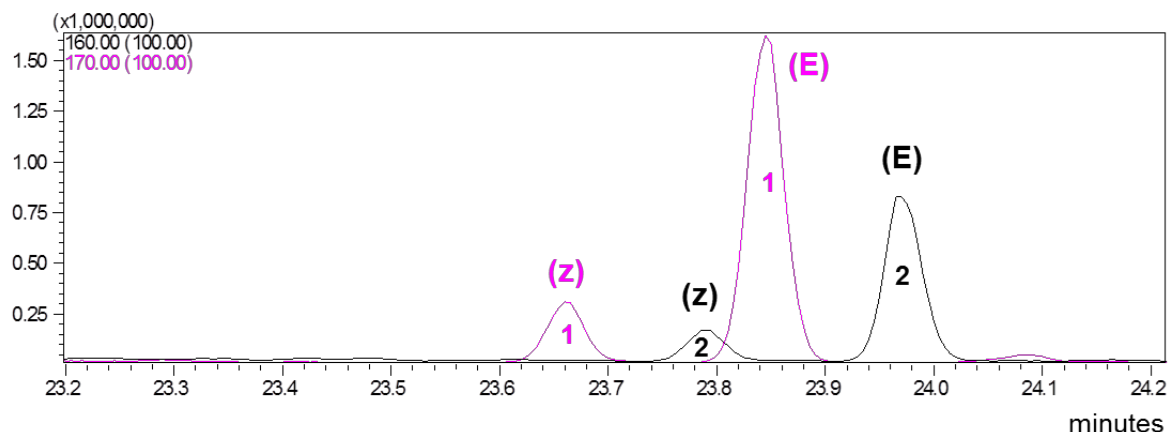


Figure 5.1. Chromatographic peaks for (Z) and (E)-4MSP methoximes for labeled (1) and unlabeled (2) 4MSP. Chromatogram was obtained from methoximation reaction with a mixture of references compounds d_{10} -4MSP at 454 ng/L and 4MSP at 240 ng/L in ethanol solution 10% (v/v) and pH 3.5.

5.3.2. Volatile thiols work-up.

Work-up begins with a liquid-liquid extraction with pentane. The objective of this solvent extraction is to get an extract enriched with mainly volatile compounds, leaving aside non-volatile components of the wine that could influence the yield of the derivatization reaction, such as phenols, which can also be measured by the PFBBBr derivatization reaction (Kuklenyik, Ekong, Cutchins, Needham, & Calafat, 2003). The extraction solvent was chosen based on its ability to avoid the emulsions commonly formed in the liquid-liquid extraction (as happens in dichloromethane extraction) and the stability of volatile thiols in that solvent. We found that the addition of small amounts of NaCl allowed the extraction to proceed without noticeable emulsion problems and the phase separations were quite easy. Additionally, it was previously reported that pentane does not promote a noticeable oxidative degradation of volatile thiols over 9 days

storage, the opposite of diethyl ether, which yields an oxidative degradation of almost 50 % for the same storage period (Hofmann et al., 1996).

The derivatization reaction with PFBBr is an $\text{S}_{\text{N}}2$ -type nucleophilic substitution with a thiolate intermediate. For this reason, alkaline conditions were needed to promote the formation of this intermediate (Lerch & Zinn, 2003). Addition of NaOH (1 M; 1 mL) gave good results for all volatile thiols analyzed, but less so for 3SHA. The latter is because NaOH is able to promote the hydrolysis of 3SHA into 3SH (Table 5.2). According to previous reports (Mateo-Vivaracho et al., 2010), the best results for the analysis of different thiols in the same chromatographic run was obtained by the addition of an aqueous DBU solution (6.7% v/v; 1 mL) to avoid any noticeable hydrolysis of 3SHA into 3SH at the derivatization conditions used in the proposed method. The novelty of the method is, the derivatization reaction was performed in an aqueous thiol solution that was obtained after liquid-liquid extraction of the wine samples with pentane, evaporation of the extract to dryness and resolution in water. The purpose of the solvent change was mainly to protect the SPME fibre used in the extraction procedure.

Finally, to enhance the sensitivity and specificity of the method and avoid matrix problems, an SPME extraction was performed according to the approach previously proposed by Capone et al. (2011) for 3MH measurements in wine samples, with slight modifications. The modifications addressed the temperature and time for extraction and desorption of the SPME fibre, principally because the chromatographic peaks were most defined and not co-eluted at these conditions (data not shown).

Table 5.2. Effect of the aqueous solution addition of NaOH (1M, 1mL) in the PFBBBr derivatization reaction of 3SHA.

	Area 3SH	Area 3SHA	Area ISTD ^d	Area ratio 3SH	Area ratio 3SHA
Assay 1 ^a	420035	- ^c	2613854	0.161	- ^c
Assay 2 ^b	2893116	- ^c	9289391	0.311	- ^c

^a Model wine solution 10% v/v ethanol at pH 3.5 spiked with 12 ng of 3MH.

^b Model wine solution 10% v/v ethanol at pH 3.5 spiked with 12 ng of 3MH and 12 ng of 3MHA.

^c No chromatographic signal detected at the retention time of the analyte.

^d Internal standard, d-3MH at 21 ng.

5.3.3. Method validation and application.

Whole method validation data is summarized in Table 5.3. According to the R^2 values, the calibration curves for all volatile thiols are linear in the interval of the calibration. Linear regressions were obtained for all thiols and built with the average values of triplicates for the five calibration points with a $RSD \leq 15\%$.

The quantitation of 4MSP and 3SH was made with their analogous deuterated forms, d_{10} -4MSP and d-3SH. Determination of the concentration of labeled compounds was achieved by GC-FID using methyl octanoate as an internal standard as described before (Sen et al., 1991). Considering that only one deuterium atom is present in the molecule d-3SH, its suitability as an internal standard needed to be tested before use. Mass spectra of labeled and unlabeled 3SH did not show any significant ion interference (Figure 5.2). However, the slope value of 0.6539 obtained for the calibration curve of 3SH, suggested a possible interference between 3SH and d-3SH. Nevertheless, validation parameters obtained for the calibration of 3SH such as repeatability, reproducibility and recovery (Table 5.3) were quite satisfactory, allowing to employ d-3SH as internal standard. The other volatile thiols, 3SHA, PhMT, and FFT, were not quantified with their respective

labeled compounds; calibration parameters such as slope, linearity, and recovery were appropriate for quantitation in wine samples.

Whole calibration was performed using a wine model solution (10% v/v ethanol, pH 3.5). However, recovery values obtained by spiking red and white wine with the volatile thiols ranged from 65 to 119%. These values are quite satisfactory and imply that the calibration parameters obtained in wine model solution are suitable for the measure of these compounds in wine samples. Quantitation of PhMT in red wine samples should be performed in duplicate and accompanied by standard addition corroboration due to its low recovery value (65%) in this matrix.

One of the most important challenges, and the main reason to develop an improved analytical method for the determination of volatile thiols, was to obtain LOD values below the odor detection thresholds determined for these compounds in wine model solution. According to the literature (Roland, Schneider, Razungles, et al., 2011), the odor detection threshold for the volatile thiols are as follows: 4MSP, 0.8 ng/L; 3SHA, 4.2 ng/L; 3SH, 60 ng/L; FFT, 0.4 ng/L; and PhMT, 0.3 ng/L. The LOD determined for the proposed method were below the odor detection thresholds for all five thiols analyzed (Table 5.3). Additionally, the proposed method was applied to the analysis of 10 wine samples, 5 red wines and 5 white wines (Table 5.4). Finally, the results obtained in real wine samples and the determined validation parameters support the idea that the analytical method proposed in the present work is suitable for measurements and analysis of volatile thiols in wine samples for oenological purposes.

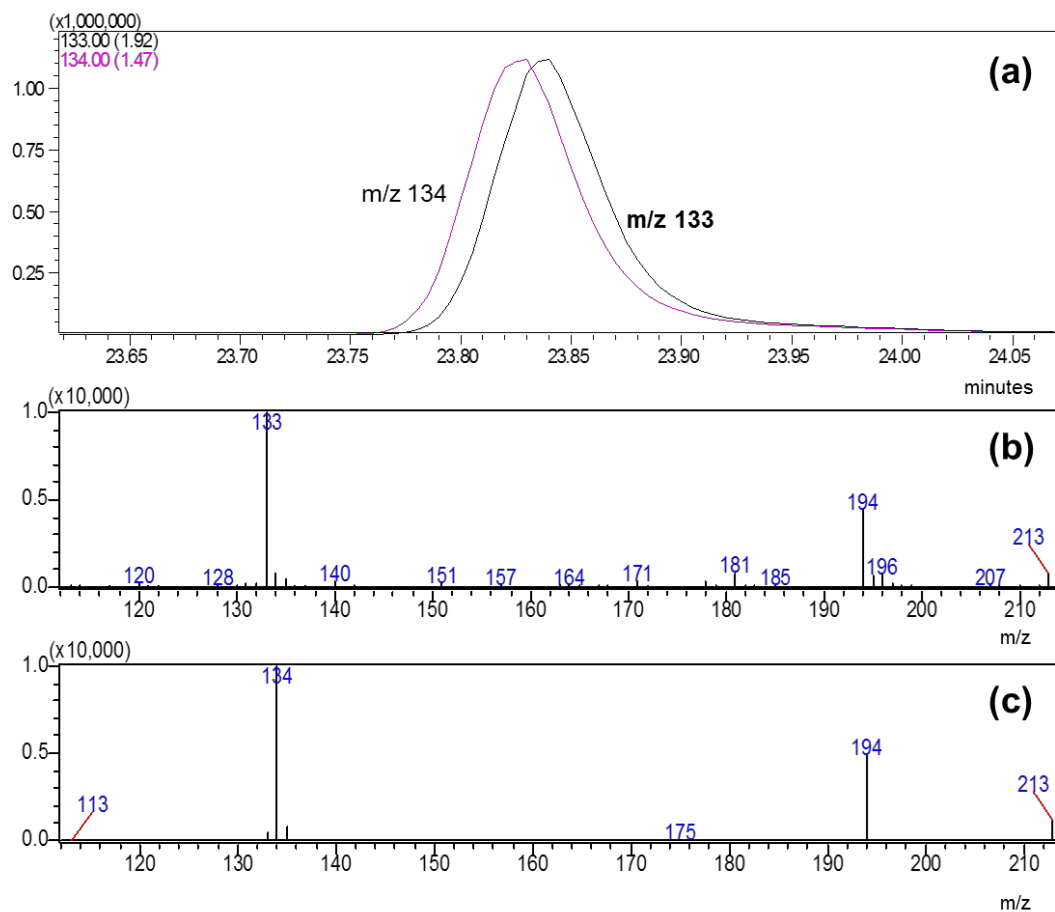


Figure 5.2. (a) Single ion monitoring chromatogram of a 1:1 mixture of PFBBBr derivative d-3SH (m/z 134) and 3SH (m/z 133). (b) Mass spectrum of PFBBBr derivative of 3SH obtained in NCI mode from the reference compound. (c) Mass spectrum of PFBBBr derivative of d-3SH obtained in NCI mode from the synthesized compound.

Table 5.3. Validation parameters for the proposed method for all volatile thiols analyzed.

	FFT	PhMT	4MSP	3SH	3SHA
Slope ^a	0.5962	1.5661	0.8001	0.6539	0.3031
Intercept	0.0714	- 0.2527	0.0162	- 0.0113	0.0012
R ²	0.9932	0.9838	0.9890	0.9982	0.9962
Recovery %					
Red wine ^b	97 ± 0.08	108 ± 0.14	119 ± 0.04	94 ± 0.04	97 ± 0.13
Recovery %					
White wine ^b	110 ± 0.07	65 ± 0.03	111 ± 0.05	102 ± 0.10	91 ± 0.05
Repeatability (RSD %)	8	1	12	9	2
Reproducibility (RSD %) ^d	8	14	4	3	13
LOD (ng/L)	0.06	0.25	0.17	0.76	1.10
LOQ (ng/L)	0.20	0.82	0.55	2.53	3.68

^a All calibration points were measured in triplicate with a RSD % ≤ 15.

^b Recovery was analysed in red and white wines to evaluate the matrix effect. Concentration recoveries in red wine for the analysed compounds were as follow: FFT 177 (ng/L); BMT 240 (ng/L); 4MMP 40 (ng/L); 3MH 2484 (ng/L) and 3MHA 680 (ng/L), and for white wine: FFT 305 (ng/L); BMT 354 (ng/L); 4MMP 360 (ng/L); 3MH 2484 (ng/L) and 3MHA 680 (ng/L).

^c Mean value of a triplicate of the same work-up performed in the same day.

^d Mean value of a triplicate of the same work-up performed in different days.

Table 5.4. Application of the method to different red and white wines.

Samples	FFT (ng/L)	BMT (ng/L)	4MMP (ng/L)	3MH (ng/L)	3MHA (ng/L)
SwW 1 ^a	n.d ^c	n.d ^c	61.9 ± 0.11	3255.9 ± 0.03	205.5 ± 0.04
SwW 2 ^a	n.d ^c	n.d ^c	9.4 ± 0.04	5131.3 ± 0.03	156.9 ± 0.02
SwW 3 ^a	n.d ^c	n.d ^c	22.2 ± 0.02	781.2 ± 0.01	78.0 ± 0.02
SwW 4 ^a	n.d ^c	n.d ^c	28.7 ± 0.12	1389.9 ± 0.11	109.8 ± 0.05
SwW 5 ^a	n.d ^c	n.d ^c	2.7 ± 0.14	1241.9 ± 0.01	55.4 ± 0.10
SrW 1 ^b	44.3 ± 0.09	46.5 ± 0.01	n.d ^c	662.0 ± 0.06	6.7 ± 0.02
SrW 2 ^b	21.0 ± 0.05	45.8 ± 0.02	n.d ^c	502.6 ± 0.02	23.7 ± 0.13
SrW 3 ^b	n.q ^d	n.q ^d	n.q ^d	2515.4 ± 0.06	27.8 ± 0.01
SrW 4 ^b	n.q ^d	90.0 ± 0.05	n.q ^d	2207.1 ± 0.13	25.3 ± 0.22
SrW 5 ^b	n.q ^d	97.4 ± 0.09	n.q ^d	3667.4 ± 0.20	29.1 ± 0.10

^a SwW: white wine samples used to perform thiols determination. All measurements were carried out in duplicate.

^b SrW: red wine samples used to perform thiols determination. All measurements were carried out in duplicate.

^c n.d: under detection limit according the values obtained in the validation method.

^d n.q: under quantitation limit according the values obtained in the validation method.

5.4. Quantitation and sensory evaluation of thiols in Carmenere red wines.

There is scarce information about the concentration range of thiols that could be found in Carmenere red wines. Therefore, 13 Carmenere wine samples with different geographical origin and year of harvest were analyzed with the optimized analytical method described above and submitted to sensory analysis.

A detailed description of the samples is provided in Table 5.5 and Figure 5.3. All samples belong to sixth region of Chile and were vinified under a traditional vinification process according to the following procedure: crushing and destemming, maceration, alcoholic fermentation and malolactic fermentation.

Table 5.5. Description of the 13 Carmenere red wines analyzed.

Nº	Origin	Harvest	Vinification	Yeast strain	Alcoholic degree
1	Peumo	2014	Traditional	IOC-18-2007	14.4
2	Peumo	2014	Traditional	F-10 ZYMAFLORE-ZIMAFLORE ALPHA	14.8
3	Peumo	2014	Traditional	D-80 LALLEMAND	14.5
4	Rengo	2014	Traditional	VI-A-DRY PDM	13.5
5	Peumo	2014	Traditional	FERMIVIN PDM	13.6
6	Peumo	2014	Traditional	PDM MAURIVIN	13.1
7	Santa Cruz	2014	Traditional	FERMIVIN PDM	13.4
8	Peumo	2014	Traditional	K1 M LALVIN	13.9
9	Quinta de Tilcoco	2014	Traditional	VI-A-DRY PDM	14.0
10	Peumo	2013	Traditional	F-15 ZYMAFLORE-UVAFER HPS	13.9
11	Peumo	2013	Traditional	FERMIVIN PDM	14.5
12	Peumo	2013	Traditional	FERMIVIN PDM - VI-A-DRY PDM	13.4
13	Rengo	2013	Traditional	VI-A-DRY PDM	13.1

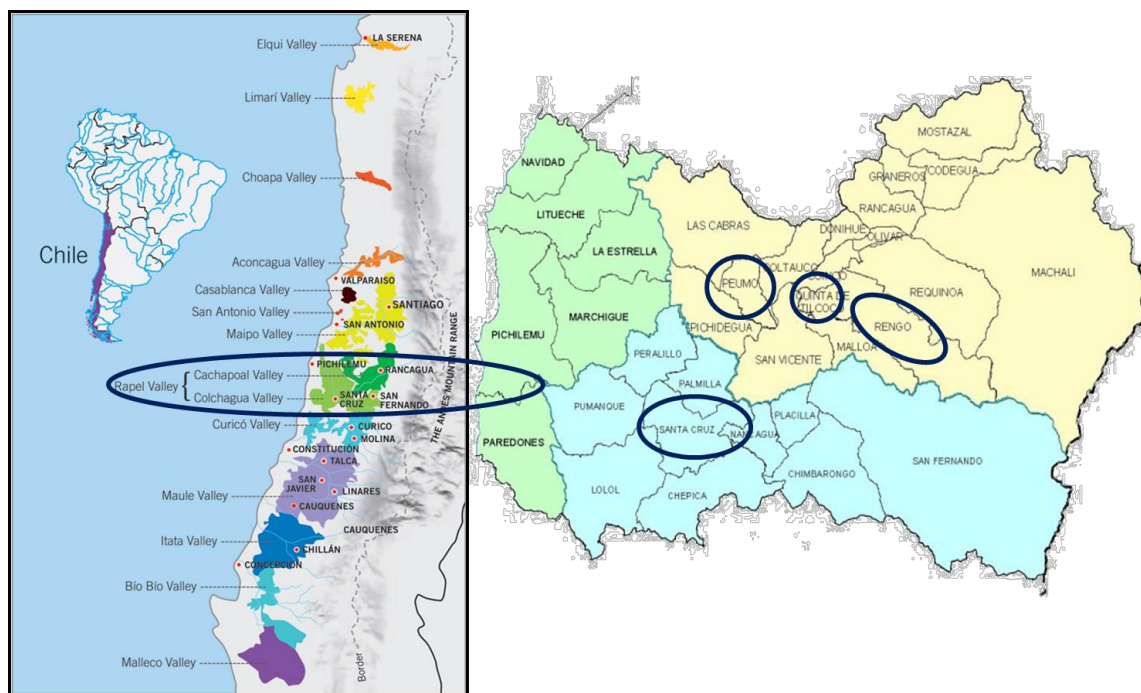


Figure 5.3. Geographical distribution of Carmenere red wine analyzed.

Results showed a wide variability in the concentrations for all thiols analyzed, especially for 3SH (Table 5.6). Concentrations ranged between 48 to 198 ng/L for BMT, 474 to 3108 ng/L for 3SH, 8.8 to 95.4 ng/L for 3SHA and 0 to 196.5 ng/L for 4MSP. The variability in the concentrations of all thiols could be explained by the different geographical origin of the samples. Indeed, some odorants, such as methoxypyrazines, are affected by geographical and climate conditions, such as slope orientation and sun exposition (Belancic & Agosin, 2007). On the other hand, alcoholic fermentation was carried out using different wine yeast strains. It has been found that it is possible to change the overall aroma of a wine with different yeasts or microorganisms during winemaking process (Swiegers et al., 2005). For instance, co-fermentation with *Pichia* spp could enhance the concentration of 3SH and 3SHA in Sauvignon blanc wines (Anfang, Brajkovich, & Goddard, 2009).

The compound FFT was identified in only one sample of 2013 harvest. It is worthy to note at this point that most of the samples analyzed are from 2014 harvest and only four are from 2013 harvest; among the latter some were stored in oak barrels during malolactic fermentation. Therefore, results obtained for FFT confirm the hypothesis that this thiol could be produced by extraction from oak barrels (Blanchard et al., 2001).

All the samples sensorially analyzed have a strong fruity aroma mainly described with the cassis, strawberry and raspberry attributes (Figure 5.4). Particularly interesting is the aroma profile exhibited by Carmenere number 13, which has a strong passion fruit aroma nuance. The latter could be related with the very high concentration of 3SH (Table 5.6). This concentration is well above the odor threshold of 3SH (60 ng/L) with an odor activity value (concentration ratio between concentration and odor threshold) of 22, strongly suggesting that 3SH could be a candidate for key aroma compound. Carmenere number 5 also exhibits an interesting aroma profile and contained the highest concentrations of thiols (3107.7 ng/L for 3SH, 95.4 ng/L for 3SHA and 65.0 ng/L for 4MSP). Paradoxically, this sample has a balanced fruity aroma profile, mainly characterized by its cassis, blackberry and raspberry nuances. The same was found for samples number 4, 8, 10 and 12 with a marked cassis aroma nuance and high concentration of thiols. Rigou et al., 2014 demonstrated for red wines that 4MSP is directly correlated with blackcurrant aromas. Additionally, compounds 3SH and 3SHA present in high concentration act as enhancers of blackcurrant aroma. Finally, there are necessary further spiking experiments in red wine matrix to elucidate the individual contribution of each thiol to fruity aromas in Carmenere red wines.

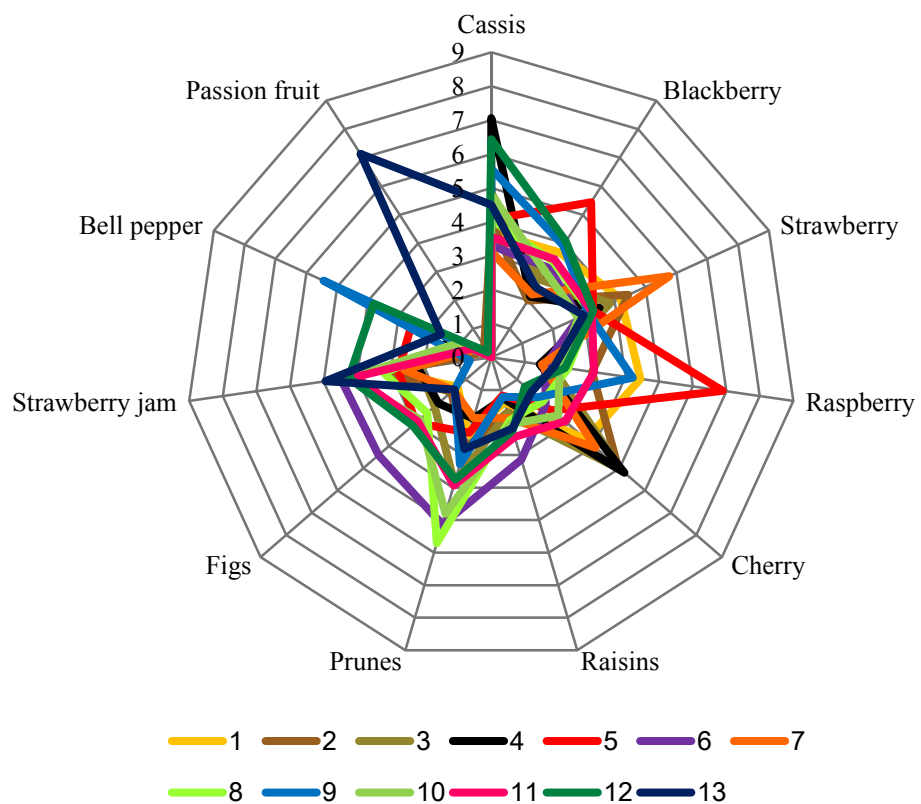


Figure 5.4. Aroma Profile of the 13 Carmenerre red wines analyzed by sensory evaluation.

Table 5.6. Concentration of thiols in the 13 Carmenere red wine samples.

Nº	Origin	Harvest	FFT (ng/L)	PhMT (ng/L)	3SH (ng/L)	3SHA (ng/L)	4MSP (ng/L)
1	Peumo	2014	NQ	70.1	728.2	19.9	196.5
2	Peumo	2014	NQ	163.3	825.1	10.3	ND
3	Peumo	2014	ND	71.9	664.3	11.0	ND
4	Rengo	2014	ND	187.5	488.3	83.5	ND
5	Peumo	2014	ND	ND	3107.7	95.4	65.0
6	Peumo	2014	ND	64.9	474.4	11.4	ND
7	Santa Cruz	2014	ND	113.4	647.8	16.0	ND
8	Peumo	2014	NQ	47.9	1150.2	38.9	NQ
9	Quinta de Tilcoco	2014	ND	75.3	824.8	32.1	ND
10	Peumo	2013	NQ	198.3	719.7	15.2	ND
11	Peumo	2013	ND	ND	604.6	8.8	ND
12	Peumo	2013	109.2	70.4	953.9	9.0	10.7
13	Rengo	2013	NQ	57.3	1303.2	10.4	ND

5.5 Conclusion

In the present work, by means of an optimization of a previously reported extraction procedure and a novel derivatization step in an aqueous reconstituted thiol solution, it was possible to isolate five volatile thiols from wine samples and quantify them in a

single chromatographic run. According to the calibration parameters, the proposed method is linear, repeatable, reproducible, and accurate at the range of concentrations studied. The LODs determined for the all five volatile thiols studied are below their respective odor detection threshold in a wine model solution. The application of the optimized technique, in combination with sensory analysis, to 13 Carmenere red wines suggests that thiols could be critical to fruity aromas of Carmenere red wine, mainly related to cassis, strawberry and raspberry nuances.

5.6. Acknowledgements

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6. Conclusions

Research on Carmenere red wine aroma compounds developed in this work, revealed twenty-one aroma-active zones in the flavor dilution (FD) factor range of 16 to ≥ 1024 . These aroma-active zones were found by application of an aroma extract dilution analysis (AEDA) to AV and NBV fractions, respectively. Most of these compounds are commonly found as aroma-active compounds in wine and are synthesized by yeast during fermentation.

However, among these 21 aroma-active compounds we identified two methyl esters not previously reported in red wines, methyl 2-methylbutanoate (M2MB) and methyl 3-methylbutanoate (M3MB). Quantitation experiments carried out in 16 different red wine samples showed the presence of these compounds in all of the samples analyzed in concentrations below the odor threshold for M2MB but close or slightly above the threshold for M3MB.

The findings discussed above confirmed the hypothesis of the existence of a wine basal aroma, mainly composed of yeast-derived aroma compounds produced during the alcoholic fermentation. Nevertheless, the identification of the two novel methyl esters in Carmenere red wine, as well as in other important red wine varieties, opens the discussion on the contribution of minor compounds to the characteristic aroma of a red wine; indeed, the latter could result from a synergistic behavior with other aroma compounds being able to influence or change the overall aroma in a red wine sample.

Additionally, we found the volatile thiols 2-furanylmethanethiol (FFT), 3-sulfanyl-1-hexanol (3SH), 3-sulfanylhexyl acetate (3SHA) and 2-methyl-3-sulfanyl-1-butanol (2M3SB) in Carmenere wine samples in aroma-active amounts. All these compounds were present in concentrations well above their odor detection thresholds. Addition of these compounds to a red wine aroma model solution, all together and each independently, were identified as different by a trained panel evaluation of the samples in a discriminant sensory analyses. Results exhibited that thiol compounds are able to influence the overall aroma of a red wine model solution. However, more sensory

experiments are needed to test the hypothesis that thiol compounds could enhance the fruity aroma notes of red wines.

It is important to mention that compound 2M3SB has been reported before only in fungal infected wines with Botrytis. As Carmenere grapes are often harvested lately in order to reduce the methoxypyrazine concentration, the latter suggests that this thiol could be an indicator of botrytis in grapes. Its odor detection threshold in synthetic wine solution (10% v/v ethanol; pH 3.5) determined for a racemic mixture was 0.4 ng/L. But it is important to keep in mind that 2M3SB is a chiral compound with four enantiomeric forms that involve four possible odorants. Therefore, further experiments are necessary for the determination of the enantiomeric ratio of these compounds in Carmenere red wines.

Finally, in the present work a new tool for the analyses of the five most important volatile thiols in winemaking (FFT, 3SH, 3SHA, 4-methyl-4-sulfanyl-2-pentanone and phenylmethanethiol) has been proposed. Validation parameters obtained for the analytical method, such as linearity, repeatability, reproducibility and recovery percentage showed that the proposed method is suitable for thiol analyses in wine samples.

7. References

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