AGRICULTURAL UNIVERSITY OF ATHENS
Department of Food Science & Human Nutrition

UNIVERSITÀ DEGLI STUDI DI UDINE
Department of Food Science

Master of Science in

Viticulture & Oenology

Postgraduate Thesis:

Effect of different fermentation management practices on the aromatic composition of Cabernet Franc wines produced in North – East Italy.

Supervisor	**Correlator**	**Postgraduate Student**
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To my parents,

Kostas & Efthimia

Περίληψη

Στην παρούσα διατριβή έγινε σύγκριση τεσσάρων διαφορετικών οινοποιήσεων, που συνδέονται με διαφορετικές πρακτικές διαχείρισης ζύμωσης, προκειμένου να αξιολογηθεί η πιθανή εναλλακτική διαδικασία βελτίωσης των αρωματικών ιδιοτήτων της ποικιλίας Cabernet Franc.

Μετά την ολοκλήρωση των ζυμώσεων που πραγματοποιήθηκαν χωρίς συμπλήρωση με διοξείδιο του θείου, ο στόχος ήταν να προσδιοριστούν και να ποσοτικοποιηθούν οι αρωματικές ενώσεις με GC-MS και να συγκριθούν οι συγκεντρώσεις τους μεταξύ των τεσσάρων οίνων. Τα ελεύθερα και δεσμευμένα τερπένια απομονώθηκαν με εκχύλιση σε στερεή φάση (SPE), ενώ οι πτητικές ενώσεις εκχυλίστηκαν με 5-DCM (2: 1, v / v). Συνολικά εννέα (9) ελεύθερα τερπένια, πέντε (5) συνδεδεμένα τερπένια, οκτώ (8) αιθυλεστέρες, μια πτητική φαινόλη, εννέα (9) ανώτερες αλκοόλες, μία λακτόνη, έξι λιπαρά οξέα και μία ένωση θείου ταυτοποιήθηκαν και ποσοτικοποιήθηκαν στα παραγόμενα κρασιά.

Τα αποτελέσματα υποδεικνύουν σαφώς ορισμένες διαφορές μεταξύ αυθόρμητης και μη αυθόρμητης ζύμωσης. Η αυθόρμητη αλκοολική ζύμωση παράγει υψηλότερη πτητική περιεκτικότητα και περισσότερα τερπένια κυρίως σεσκιτερπένια. Ο συνδυασμός μη αυθόρμητης αλκοολικής και μη αυθόρμητης MLF ζύμωσης έδωσε τα καλύτερα αποτελέσματα όσον αφορά την ένταση φρουτώδους αρώματος και δεν παρήγαγε οξικό οξύ. Επίσης, διαπιστώθηκε ότι μεταξύ της αυθόρμητης και της μη αυθόρμητης μηλογαλακτικής ζύμωσης υπάρχουν σημαντικές διαφορές. Ο συνδυασμός μη αυθόρμητης αλκοολικής και αυθόρμητης MLF ζύμωσης παρουσίασε την ελάχιστη πτητική περιεκτικότητα.

Είναι προφανές ότι ο εμβολιασμός με επιλεγμένους ζυμομύκητες και γαλακτικά βακτήρια επηρεάζει τη σύνθεση αρώματος του κρασιού και τις αισθητικές ιδιότητες, αλλά για την ενίσχυση αυτής της πληροφορίας θα πρέπει να πραγματοποιηθούν περαιτέρω πειράματα μεταξύ αυθόρμητων και τη μη αυθόρμητων ζυμώσεων.

Λέξεις Κλειδιά : Μηλογαλακτική ζύμωση, αλκοολική ζύμωση, μη αυθόρμητη, αυθόρμητη, φρουτώδες άρωμα, εκχύλιση, αρωματικές ενώσεις,GC-MS.

Abstract

In the present thesis was to compare four different vinification, connected with different fermentation management practices in order to evaluate possible alternative process to improve the aromatic qualities of the variety Cabernet Franc.

After the completion of fermentations, which carried out without sulfur dioxide supplementation, the objective was to identify and quantify aroma compounds by GC-MS, and to compare their concentrations between of the four wines. Free and bound terpenes were isolated by solid phase extraction (SPE), while volatile compounds extracted with 5-DCM (2:1, v/v). A total of nine (9) free terpenes, five (5) bound terpenes, eight (8) ethyl esters, one volatile phenol, nine (9) higher alcohols, one lactone, six (6) fatty acids and one sulfur compound were identified and quantified in the produced wines.

The outcomes clearly indicate some differences between spontaneous and non-spontaneous fermentation. The spontaneous alcoholic fermentation produces higher volatile content and more terpenes mainly sesquiterpenes. The combination of non-spontaneous alcoholic and non-spontaneous MLF fermentation gave the best results concerning of intensity of fruity aroma and did not produce acetic acid. Also, it was found that between nature and non-spontaneous MLF fermentation there are important differences. The combination of non-spontaneous alcoholic and spontaneous MLF fermentation presented the least volatile content.

It was obviously that the choice of yeast and bacteria inoculums, affects wine aroma composition and sensory properties but further experiments will be useful to enhance this information between the differences in spontaneous and non-spontaneous fermentation.

Key words: Malolactic fermentation, alcoholic fermentation, non-spontaneous, spontaneous, fruity aroma, extraction, aromatic compounds, GC-MS.

Acknowledgement

The research work of this thesis was carried out at University of Udine (Università degli Studi di Udine). I take this opportunity to express my deep sense of gratitude to the people who have been instrumental in the successful completion of this thesis.

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Similar, profound gratitude goes to others faculty and staff members of the laboratory for their kind co-operation and to all staff members of the Erasmus offices, both in Greece and Italy, for their crucial help.

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Chapter 1

Introduction

1.1 Historical Background

Wine is one of the foundations of Western civilization. The story of wine is that of religion, medicine, science, war, discovery and dream. Who made the very first wine and precisely where will probably never be established. However, it is generally considered that vines originated from the Caucasus area of Russia, between the Baltic and the Caspian Seas *(Johnson, 1989 & McGovern, 2003)*. The recent discovery of a 6.000-year-old winery in a cave in Armenia with all the necessary equipment, including a grape press, fermentation vats and storage jars, confirms the area had the technology to produce wine (Fig.1) *(Barnard et al., 2011)*.

Figure 1: Entrance to the Areni-1 cave in southern Armenia near the town of Areni *(https://en.wikipedia.org/wiki/History_of_wine)*.

The tartaric acid residue on 8.000-year-old pottery fragments in Greece *(Keys, 2003)*, the well-preserved painting in the tomb of Nakht, illustrating grape harvest and wine making *(Egyptian Tourist Bureau, 2011)*, the creation of the Greek God Dionysus and the Roman God Bacchus representing the god of wine and the references to grapes and wines in the classical Greek and Roman writings show the presence of wine in the lives of people in the ancient world *(Clinkerbread, 1982)*.

After the decline of Greek and Roman civilizations, wine production in Europe established late medieval times. In the late nineteenth century wine production was fully established in France *(J. Bakker & R.J. Clarke, 2012)*. In the 16th century, the first Spanish conquistadors brought the European grape varieties to the region where is now Mexico *(Dickenson & Unwin, 1992)*. Wine production began in the Cape Province of what is now South Africa in the 1680s. Australia's First Fleet (1788) brought cuttings of vines from South Africa, although initial plantings failed and the first successful vineyards were established in the early 19th century *(De Blij & Harm, 1985d)*.

In the 1800s the Europe's vineyards were devastated by many diseases, but the main afflict the vines was Phylloxera. This is an insect which attacks the roots of the plants. During the disaster, was found that Native American vines were immune to the pest and all the vineyards in the world replanted, with (selected) v. vinifera cultivars grafted onto American rootstock *(Cazalis, 1869a)*. The quality of these early wines was low by today’s standards. The improvement was done with the scientific achievements of Pasteur in the 1860s. The discovery of the microbiological processes involved in wine-making laid the foundations for the modern wine industry *(Stefan K. Estreicher, 2006)*.

Today, France, Italy and Spain are still the three largest wine producers in Europe. Wine in the America is often associated with Argentina, California and Chile, all of which produce a wide variety of wines. Australia, New Zealand, South Africa and other countries without a wine tradition are considered New World producers. With the increase in mechanization and scientific advances in winemaking, these countries became known for high-quality wine *(Banks et al., 2007)*.

1.2 Cabernet Franc

Cabernet Franc is an old French variety and one of the major black grape varieties all over the world. Cabernet Franc is believed to have been established in the Libournais region of southwest France in the 17th century. It is a parent to Cabernet Sauvignon (the other is Sauvignon Blanc) and presents many of the same phenolic and aroma compounds but with some noticeable differences. Cabernet Franc tends to be more lightly pigmented and produces wines with the same level of intensity and richness. Also, it tends to have a more pronounced perfume with notes of raspberries, blackcurrants, violets and graphite. It is often characterized by a green, vegetal strike that can range from leaves to green bell peppers. Cabernet Franc has slightly less tannins than Cabernet Sauvignon and tends to produce a wine with a smoother mouthfeel. It is a useful grape in many blends with Cabernet Sauvignon and Merlot *(Manolis N. Staurakakis, 2010)*.

The Cabernet Franc plants thrive in fertile soil, deep, cool, different compositions and well drained hilly, upland or mountain areas. It is cultivated in Australia, South Africa, Chile, Argentina, New Zealand, USA, Canada, Hungary, Spain, Bulgaria, Slovenia, Croatia and Italy. In the countries of "New World" tend to produce wines with more emphasis on the fruit character than the green notes. In Greece is cultivated to the region of Peloponnese, Viotia, Drama, Ioannina, Kavala, Serres and Chalkidiki *(Manolis N. Staurakakis, 2010)*.

1.3 Red Winemaking

Wine is fermented grape or grape juice. There are three types of wines, white, red and rosé wines. The red color comes from anthocyanin pigments present in the skin of the grape. In conventional red winemaking, extraction of grape skins, seeds and possibly stems, is by means of maceration, which occurs during must fermentation. Other methods exist that dissociate fermentation and maceration, such as thermovinification *(E. Soufleros, 2000)*.

Red wine is made from juice and skins of red varieties and white wine is normally made from white varieties. The localization of red pigment exclusively in skins permits a slightly tinted or white wine to be made from the colorless juice obtained from a delicate pressing of red grapes *(Sacchi et al., 2005)*. Varietal nature is not sufficient for characterizing the origin of a red wine. Maceration length and intensity are of prime importance.

There are three main phenomena occurring during of red winemaking *(E. Soufleros, 2000)*:

- Alcoholic fermentation
- The extraction of red pigment (maceration) and
- Malolactic fermentation

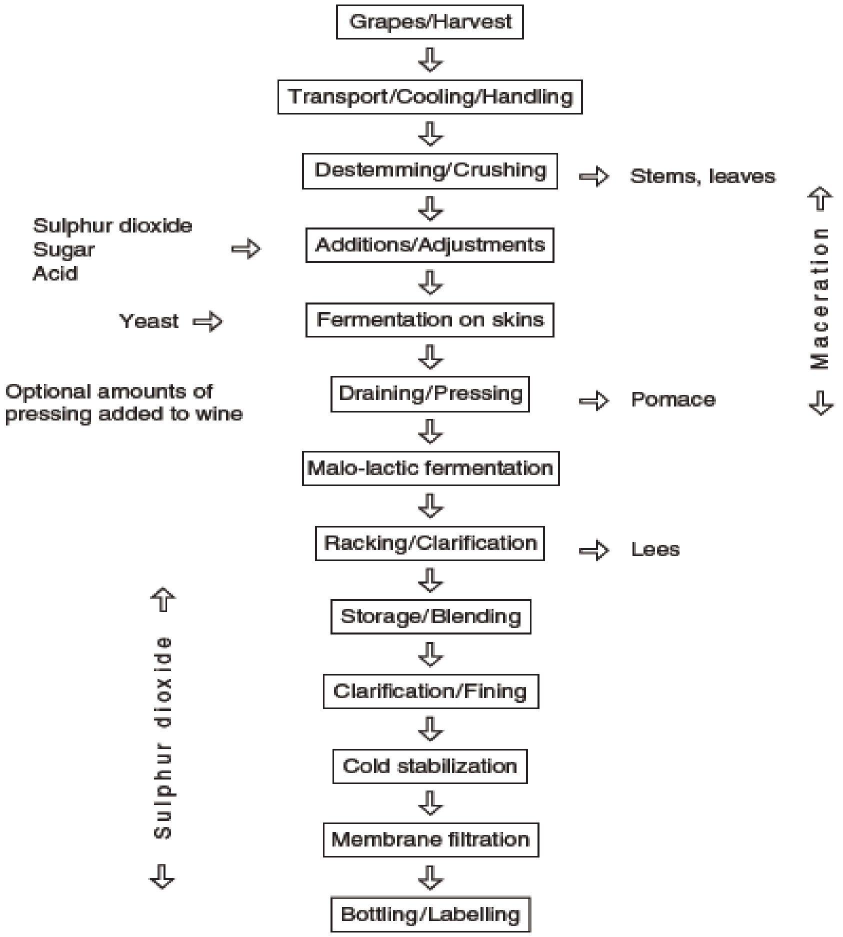


Figure 2: Typical processing sequence in vinification of red wines *(J. Bakker & R J. Clarke, 2012)*.

The involved phenomena in red winemaking are (Fig. 2) *(E. Soufleros, 2000)*:

- mechanical harvest treatments (crushing, destemming and tank filling),
- vatting (primary alcoholic fermentation and maceration),
- draining (separation of wine and pomace by dejuicing and pressing),
- final fermentations (exhaustion of the last grams of sugar by alcoholic fermentation and malolactic fermentation).

1.4 Fermentation

Fermentation is a metabolic process that converts sugar to acids, gases, or alcohol. It occurs in yeast and bacteria, and also in oxygen-starved muscle cells, as in the case of lactic acid fermentation. The word "fermentation" is derived from the Latin verb "fervere", which means to boil *(E. Soufleros, 2000 & Ronald S. Jackson, 2008)*.

The process of fermentation in winemaking turns grape juice into an alcoholic beverage. There are two kinds of fermentation in winemaking, alcoholic fermentation and malolactic fermentation. During alcoholic fermentation, yeasts transform sugars present in the juice into ethanol and carbon dioxide (as a by-product) *(J. Robinson, 2006)*. During malolactic fermentation, bacteria convert malic acid into lactic acid *(Boulton et. al, 1996).* Fermentation can be done in stainless steel tanks, in an open wooden vat, inside a wine barrel and inside the wine bottle itself as in the production of many sparkling wines *(Ronald S. Jackson, 2008)*. The duration is from 5 to 14 days for primary fermentation and potentially another 5 to 10 days for a secondary fermentation.

1.4.1 Alcoholic Fermentation

The low pH of wine, high sugar content, rapidly generated anaerobic conditions and presence of phenolic compounds create the ideal environment to support the growth of yeasts and to enrich these organisms over other microbes *(Renouf et al. 2007)*. Yeasts used in winemaking generally belong to the *Saccharomyces genus* and the most important specie of which is *S. cerevisiae* *(Ronald S. Jackson, 2008)*. *Saccharomyces* can be detected on grape surfaces, but is present at very low levels *(Prakitchaiwattana et al., 2004 & Martini et al., 1996)*. There are three principal genera found on grapes: *Hanseniaspora uvarum, Metschnikowia pulcherrima,* and *Candida stellata* *(Combina et al., 2005 & Hierro et al., 2006 & Clemente-Jimenez et al., 2004)*. The presence of yeast genera depends upon regional and climactic influences, disease pressure and level of damage of the grapes, the grape variety and vineyard. *Saccharomyces* is more commonly isolated from heavily damaged grapes *(Mortimer & Polsinelli, 1999)*.

Alcoholic fermentation can be divided into two types: directly inoculated (non-spontaneous) and uninoculated (spontaneous). Uninoculated fermentations rely on the indigenous flora of the grapes. In the case of inoculated fermentations, a strain of *Saccharomyces cerevisiae* added to the must. Inoculate yeasts need to be rehydrated in "starter cultures". The exact amount of freeze-dried culture varies by manufacturer and strain of yeast but it is often around 1g/hl *(E. Soufleros, 2000)*.

In both cases, following crushing of the grapes, the must generally displays high concentrations of the yeasts present on the grape berry *(Clemente-Jimenez et al., 2004 & Fleet et al., 2002 & Schuller et al., 2005)*. These yeasts initiate the bioconversion of grape juice into wine. How long the non- *Saccharomyces yeasts* persist depends upon the winemaking conditions and relative levels of the major species present. As fermentation progresses, the levels of these yeasts decrease, while that of *Saccharomyces* increase *(Fleet & Heard, 1993)*. By the end of fermentation, *Saccharomyces* is the majority of the yeasts found, and often the only yeast isolated. Inoculation with *Saccharomyces* leads to a faster domination of the fermentation and more rapid inhibition of the other yeasts present *(Ganga & Martinez, 2004 & Egli et al., 1998)*.

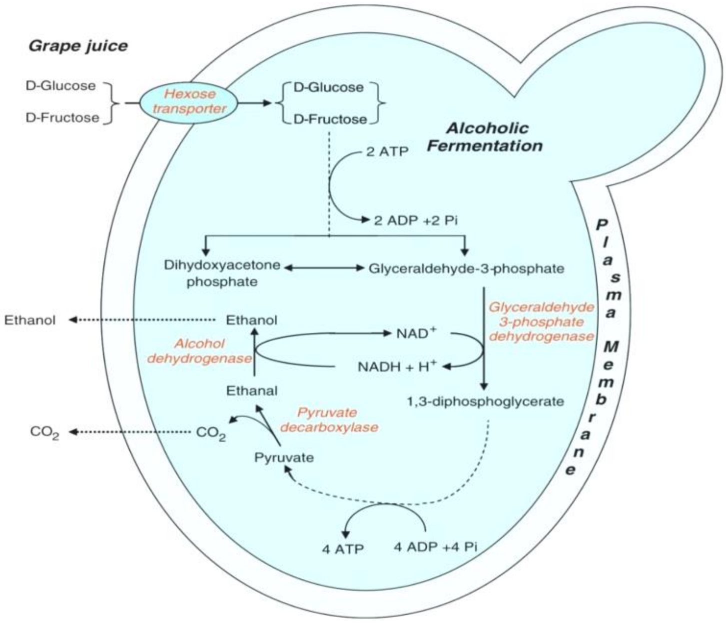


Figure 3: Alcoholic Fermentation Pathway.

The alcoholic fermentation carried out with two reactions. First effected decarboxylation of pyruvic acid (pyruvate), which is originated from glycolysis, into acetaldehyde and then this is reduced into ethanol by NADH. The second reaction is catalyzed by the alcohol dehydrogenase (Fig.4) *(Neuberg, 1946)*. Glycolysis, conversion of glucose into pyruvate, is carried out entirely in the cytosol of the *Saccharomyces cerevisiae* cell (Fig.3). Also, *Saccharomyces cerevisiae* pyruvate decarboxylase (PDC) comprises two isoenzymes: a major form, PDC1, representing 80% of the decarboxylase activity, and a minor form, PDC5, whose function remains uncertain *(Caubet et al., 1988)*.

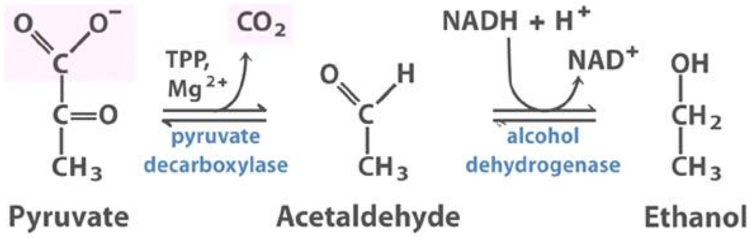


Figure 4: Decarboxylation of pyruvic acid into ethanol.4e

In winemaking, the main objective is to optimize product quality and the alcoholic fermentation is a vitally important stage on this aim. The yeast not only converts sugar to alcohol but also produce esters, aldehydes, acids and other compounds, which contribute to the wine flavor. In the last 30 years, the wine industry has tended to use the non-spontaneous fermentation. The most wines are produced using selected commercial strains of *Saccharomyces sp* and even small wineries select yeasts from their own environment for use as starter cultures.

More than 200 different *S. cerevisiae* strains are currently available commercially, with highly diverse fermentation properties. The choice of strain used by the winemaker is increasingly motivated by the potential impact of that strain on the wine characteristics. Many studies showed that specific strains are useful for increasing the fruity character, for improving some varietal characters, for limiting the production of organic acids or increasing the production of glycerol, for producing mannoproteins, for improving the color of red wines through their interactions with polyphenolic compounds, for increasing the expression of varietal characters by the hydrolysis of glycoside-bound volatile compounds and for limiting off flavors, including those due to sulphur and volatile phenols *(Dubourdieu et al., 2006 & Eglinton et al., 2000 & Ugliano et al., 2006 & Rauhut, 1993 & Shinohara et al., 2000 & Medina et al., 2005 & Moine-Ledoux & Dubourdieu 2002)*.

On the other hand, many authors report that they feel that spontaneous fermentation promotes better mouth feel in wines. That is, the wines are thought to be softer and creamier than those made using single strain starter cultures. This suggestion however has not been conclusively demonstrated scientifically.

Temperature and speed of fermentation are important considerations as well as the levels of oxygen present in the must at the start of the fermentation. Red wine is better to ferments at 24–28°C (no higher than 30°C) for up to two weeks. Higher fermentation temperatures for red wines tend not to produce the fruity fermentation esters. Generally higher fermentation temperatures increase the phenolic extraction *(Sacchi et al., 2005)*. On the other hand, white wine is fermented between 15 - 20°C giving a greater concentration of esters (fruity character).

1.4.2 Malolactic Fermentation

Malolactic fermentation is a secondary wine fermentation, which often follows the alcoholic fermentation, but can sometimes run concurrently with it. Malolactic fermentation carried out by the malolactic bacteria and the word "malolactic" comes from the conversion of L-malic acid into L-lactic acid. Actually, malolactic bacteria are capable of direct decarboxylation of malic acid to lactic acid by the enzyme malate carboxylase, which is present in various lactic acid bacteria, but particularly in three genera: *Lactobacillus, Leuconostoc* and *Pediococcus*. During this conversion, CO_2 is also produced (Fig.5) *(T.Faruk Bozoglu & Seyhun Yurdugul, 1999)*.

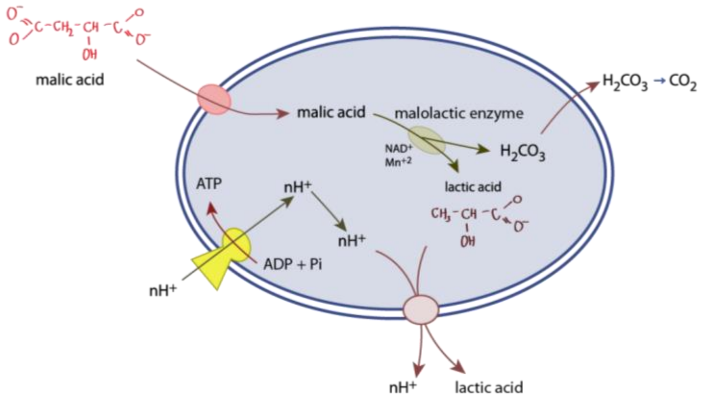


Figure 5: Malolactic Fermentation *(http://balsamorhiza .org/2014/10/ malolactic_fermentation/)*.

Lactic acid bacteria (LAB) can be found on grapes, in grape must and wine. Undamaged grapes contain <10^3 CFU/g and the initial titer in must is low *(Lafon-Lafourcade et al., 1983)*. Malolactic fermentation is commonly practiced in red wines and can be allowed to take place by naturally occurring lactic acid bacteria (spontaneous fermentation) or by specially prepared inoculums (non-spontaneous fermentation). The acidic conditions (pH: 3.0–3.5) and ethanol concentrations in grape must provide a suitable natural habitat only for a few LAB species of the genera *Lactobacillus, Leuconostoc, Pediococcus, Oenococcus* and *Weissella*. The malolactic fermentation mainly carried out by bacteria *Oenococcus oeni*, which can grows up in conditions of pH <3.5, level ethanol > 10 vol% and SO_2 concentration ~50mg / l *(Coucheneyet et al., 2005)*. For malolactic fermentation is preferred a temperature around 20°C. During the first days of must fermentation the CFU of LAB increases from 10^2 to 10^4–10^5 / ml. After the alcoholic fermentation and during the malic acid fermentation, the cell number can reach a titer of 10^7–10^8 CFU /ml *(Ribérau-Gayan et al., 2006a, b)*.

In addition, lactic acid bacteria metabolize tartrate and citrate acid. Citric acid is present in only small amounts in grapes and in wine, unless added. Citrate is converted to lactate, acetic acid, CO_2 and acetoin. Tartrate can be converted to lactate, acetate and CO_2 by the homofermentative lactic acid bacterium *Lb. plantarum* and to acetate and CO_2 or fumaric acid (succinic acid) by the heterofermentative lactic acid bacterium *Lb. brevis (Radler & Yannissis, 1972)*. One more product of LAB is the biogenic amines and the most important is histamine *(Landete et al., 2005 & Mangani et al., 2005)*. Biogenic amines can cause health problems *(Coton et al., 1998)* and sensory defects in wine *(Lehtonen, 1996 & Palacios et al., 2004)*. *Oenococcus oeni* tends to produce the least amount of biogenic amines. Also, lactic acid bacteria can produce acetic acid, diacetyl, acetoin, 2,3-butandiol, ethyl lactate, diethyl succinate and acrolein *(Boulton et al., 1996)*. Many authors observed that the MLF often produces the reduction of vegetable and herbaceous aromas and the appearance of other fruity, floral, nutty or milky aromas *(Maicas et al., 1999 & Palacios et al., 2003)*. Consequently, malolactic fermentation is very important for the winemakers because it influences aroma, quality, consistency and safety of wine *(Boulton et al., 1996)*. General, during action of lactic acid bacteria is observed:

- decrease in titratable acidity and increase in pH,
- a “gentle” flavor of wine,
- increase in fruity/butter character and reduce vegetable odor,
- decrease in color up to 30%,
- biological stabilization of wine.

In industrial processes, the development of malolactic fermentation starts immediately after alcoholic fermentation and the results can be different to those obtained in the laboratory. The success of MLF is not always guaranteed and the addition of a starter culture can improve the viability. Overall, two possibilities of lactic acid bacteria inoculation exist: traditional inoculation after alcoholic fermentation (sequential), or simultaneous inoculation in the must with yeast (co-inoculation).

It is well known that co-inoculation at the beginning of alcoholic fermentation, has several clear benefits compared to the sequential technique. The first advantage is that the introduction of lactic acid bacteria helps the bacteria to adapt to the medium better. Secondly, the contents of some compounds that are known to inhibit lactic acid bacteria growth, such as ethanol and SO_2, are lower and the medium is richer in nutritive elements during the first hours of alcoholic fermentation than at the end *(Rosi et al., 2006 & Jussier et al., 2006 & Massera et al., 2009 & Knoll et al., 2012 & Pan et al., 2011)*. In a recently study done in high alcohol wines showed that in Corvina and Rondinella varieties, which are used in the production of Amarone wine, the acetic acid levels were similar, or even lower in co-inoculation situation compared to sequential inoculation.

One of the more obvious advantages of co-inoculation is a better control over the winemaking process in terms of time management and security of MLF completion. Co-inoculation reduces the total fermentation time. This reduction limits the risk of spoilage by other microorganisms, such as the Brettanomyces species, which are mainly responsible for production of ethylphenols and vinylphenols *(Curtin et al, 2007)*.

General, co-inoculation is a tool that can be used to ensure problems normally associated with some sequential inoculations are no longer part of the equation, as well as to diversify your wine style through the production of different aroma compounds or ratios of aromas in the final wine. This technology has also opened the opportunity for other wine LAB, such as *Lactobacillus plantarum* to be used in the future as MLF starter cultures, as the matrix and challenges are much less compared to sequential inoculation *(Lerm et al., 2011)*.

Chapter 2

Wine Aroma

2.1 Volatile Compounds

Consumers want many things when drink wine, such as wine balance or harmony, good of color and touch, good flavor, etc. Among the factors that contributed to the typicality and quality of wine, aroma is probably the most important organoleptic characteristic *(Diez et al., 2004 & Selli et al., 2004)*. The aromatic compounds are either derived from grapes (Primary aroma), whether produced during alcoholic fermentation (secondary aroma), or are formed after the end of fermentation, enzymatically or chemically (tertiary aroma) *(Rapp, 1998)*. Obviously the wine aroma depends on interactions of many factors, such as climate, vineyard geographical site, viticultural practices, winemaking techniques, yeast strain, and grape variety *(Bureau et al., 2000 & S. Koundouras et al., 2006 & C. Armanino et al., 2008)*. The highest percentage of aromatic compounds produced by yeast during fermentation *(Fleet, 1996)*.

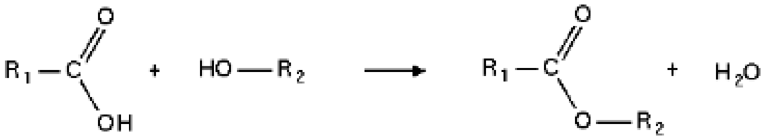
The aromatic compounds in wine classified into two groups, those that make up the fragrance for young wines and those that make up the "bouquet". The aroma of young wines consists of components emitted directly from the grapes and others formed during fermentation, while "bouquet" is the compounds formed after the end of fermentation or during aging *(E. Soufleros, 2000)*. In addition, the aroma of wine could be classified into the free or bound aroma because aromatic compounds in must and wine are found in free or in bound form, usually glycoside *(Williams et al., 1982 & Hjelmeland A.K. et al., 2014)*. Particularly, during winemaking, the “free” aroma compounds are released as a result of physical crushing and subsequent chemical and enzymatic hydrolysis of the conjugated volatiles by grape, yeast, and/or industrial enzymes (glycosidases or peptidases).

Many studies have indicated that the volatile compounds are the responsible for the aromatic character of wine. Thus far, over 1.000 volatile compounds have been identified in wines, including higher alcohols, aldehydes, esters, fatty acids, ketones, monoterpenes, volatile phenols etc. *(Rapp & Mandery, 1986)*, with a wide concentration range varying from hundreds of mg/L to the µg/L or ng/L level *(Ebeler, 2001 & Gómez-Míguez et al,. 2007 & Marti et al., 2003)*.

It is fact that not all volatile compounds affect the overall wine aroma, some of them are responsible for odors, whereas others might not produce noticeable odors at all *(Simpson & Miller, 1984)*. A comparison of aroma compounds in different wines concluded that there were concentration differences of some volatiles among the wines. The most significant differences are quantitative rather than qualitative *(Lopez et al., 1999 & Ferreira et al., 2000)*. To estimate the contributions of the volatiles in wine, the odor active value (OAV) was introduced. It is the ratio between the measurement of the concentration of volatiles in wines and their odor threshold. The contribution of volatiles to the final aroma depends on whether their concentration in the wine is above the perception threshold *(*Tao Y.S. & Zhang L., 2010*)*. Only those odorants with an OAV > 1 can be perceived *(Vilanova & Martinez, 2007 & Guth, 1997 & Li, 2006)*.

2.1.1 Esters

Esters result from the combination of organic alcohols with organic acid with the elimination of water.



$$R_1-COOH + HO-R_2 \longrightarrow R_1-COO-R_2 + H_2O$$

More than 160 esters have been isolated from wine *(Montedoro & Bertuccioli, 1986)*. Generally esters in wines can be divided in two groups. The first group consists of acetate esters, in which the acid group is derived from acetic acid and the alcohol group is ethanol or a complex alcohol (higher alcohols). The second group is the ethyl esters, where the acid group is a medium chain fatty acid and the alcohol group is ethanol.

Esters of all kinds are regarded as especially important to wine flavor, and are usually secondary aromas, arising from the fermentation, and sometimes tertiary aromas arising from ageing. It is generally recognized that the lower aliphatic ethyl esters (C_2 - C_7) show fruity notes (tropical fruit, banana, pineapple, apple, pears, etc.), whereas the higher homologues tend towards soapy, oily and candle characteristics *(Vernin et al., 1986 & Marais et al., 1979)*. Other esters, such as ethyl benzoate, iso-amylacetate and hexyl acetate, will also show important fruity characteristics. Table 1 gives some reported odor characteristics of esters in wine.

Table 1: Odor characteristics of some esters in wines *(J. Bakker & R.J. Clarke, 2012)*.

Esters	Odor
3-Methyl-butyl acetate 3-Methyl -butyl (isoamyl)	Banana
Benzyl acetate Pentyl (n-amyl) acetate	Apple
Ethyl methanoate (formate)	Ethereal, Fruity, Like Rum
Ethyl acetate	Acetone odor /Ethereal, Fruity [at low (100 ppm) concentrations]
Ethyl 2-methyl propanoate (isobutyrate)	Apple, Banana, Pineapple, Herbaceous
Ethyl propanoate (propionate)	Sweet, Ethereal, Fruity, Like Rum
Ethyl n-butanoate (butyrate)	Ethereal, Pineapple, Banana, Buttery
Ethyl 3-hydroxy-butanoate	Fruity, Green
Ethyl 2-methyl pentanoate Ethyl 4-methyl pentanoate	Fruity, Anise
Ethyl 3-methyl - pentanoate/ butanoate	Fruity
3-methyl butyl - butanoate/hexanoate/ octanoate/ decanoate	Apple, Banana
Ethyl cyclohexanoate	Licorice, Anise
Ethyl n-hexanoate	Flower, Apple, Banana, Peach
Ethyl-2-methyl butanoate	Apple, Banana, Pineapple
Ethyl octanoate	Soapy, candle
Ethyl decanoate/ Ethyl dodecanoate	Oily, Fruity, Floral
Ethyl nonanoate	Oily, Nutty
Ethyl cinnamate	Honey, Flower, Fruity
Ethyl mono-hydroxy propanoate	Ethereal, Buttery,

2.1.2 Alcohols

The most significant aromatic alcohols in wine are higher alcohols. These are alcohols with more than two carbon atoms and often constitute about 50% of the volatile aroma compounds in wine, excluding ethanol. Typical concentrations in wine are 150–550 mg/L *(Ribéreau-Gayon et al., 2006)*. The higher alcohols from grapes that survive fermentation are hexanols, 2-ethyl-1-hexanol, benzyl alcohol, 2-phenylethanol, 3-octanol, and 1-octen-3-ol. Hexanol has a “grassy” odor that is reflected in the flavor of wines. However, the most higher alcohols found in wine are the by-products of yeast fermentation. They may originate from grape-derived aldehydes, by the reductive denitrification of amino acids, or via synthesis from sugars *(Chen, 1978)*. Additionally, higher alcohols may come from the metabolic activity of bacteria. An example is the mushroom alcohol (1-octen-3-ol), synthesized during noble rotting by *Botrytis cinerea* *(Rapp & Güntert, 1986)*.

The most important higher alcohols are the straight-chain alcohols – 1-propanol, 2-methyl-1-propanol (isobutyl alcohol), 2-methyl-1-butanol, 3-methyl-1 butanol (isoamyl alcohol) and 2-phenylethanol *(Rapp & Mandery, 1986)*. Most straight-chain higher alcohols have a strong pungent odor. At low concentrations (≤ 0.3 g/L), they generally add an aspect of complexity to the bouquet. At higher levels, they increasingly overpower the fragrance. 2-Phenylethanol has a positive effect on wine flavor. This compound has a rose odor and is a key component of volatile Muscadine wines *(Bruno M. et al., 2005 & Forster H. et al., 1980)*. Finally, glycerol and 2, 3-butanediol (2,3-butylene glycol) impacting on taste of wine giving a bitter feeling *(Forster H. et al., 1980)*.

2.1.3 Terpenes

Terpenes are composed of two, three, four, or six isoprene units (2-methyl 1, 3-butadiene) and called monoterpenes (10 carbon atoms), sesquiterpenes (15 carbon atoms), diterpenes and triterpenes, respectively. They are primarily derived from grapes and exist in three forms: free monoterpene alcohols or oxides, glycosides (bound with sugars) and di- or triols *(Strauss et al., 1986 & J.J. Mateo et. al., 2000)*.

Only monoterpenes and sesquiterpenes contribute to wine fragrance and provide the characteristic odor of many flowers, fruits, seeds, leaves, woods, and roots. Monoterpenes occur in the form of simple hydrocarbons (limonene, myrcene, etc.), aldehydes (linalal, geranial, etc.), alcohols (linalol, geraniol, etc.), acids (linalic and geranic acid, etc.), and even esters (linalyl acetate, etc.). Odoriferous terpenes are known as free terpenes (unbound to sugars). The most important monoterpenes in wine are linalool (rose, citrusy), geraniol (rose, floral), citronellol (citronella), ho-trienol (linden), a-terpineol (lily of the valley, lilac), limonene (citrusy,oranges) and nerol (rose, floral) *(Ribıreau-Gayon et al., 2006 & Wood et al., 2008)*.

Glycosylated forms are known as bound terpenes and consist of the main monoterpenoids and terpene polyols including basic ‘oses’: glucose, arabinose, rhamnose and apiose. Four types of glycosides have been identified: three diglycosides (6-O-α-L-arabinofuranosyl-β-D-glucopyranoside, 6-O-α-L-rhamnosyl-β-D-glucopyranoside or rutinoside, 6-O-β-D-apiosyl-β-D-glucopyranoside) and one monoglucoside (β-D-glucopyranoside) *(Williams et al., 1982 & Gunata, 1984 & Voirin et al., 1990)*. Although, glycosides are not aromatic compounds are important to wine fragrance, because they are hydrolyzed by enzymes (*β*-glycosidases) releasing the corresponding odoriferous aglycone *(Bayonove et al., 1984 & Ayran et al., 1987 & Biron et al., 1988 & Gunata et al., 1989)*. All grape varieties contain similar glycosides, but the Muscat-flavored grape varieties have the highest concentrations.

Terpenes contribute to the varietal character of several important grape varieties, such as the presence of three monoterpene alcohols (linalool, α-terpineol and geraniol) in Muscat grapes *(Cordonnier,1956 & Rapp, 1998)* and the sesquiterpene, rotundone, recently identified as the source of the peppery aroma that frequently characterizes the aroma of Shiraz and possibly some Cabernet Sauvignon wines *(Wood et al., 2008)*. General, terpenes compounds associated with the floral aroma of aromatic grapes, such as Malvaceae, Gewürztraminer, Pinot Gris, Riesling, Viognier, Muscadelle etc. *(Rapp et al., 1978)*.

Although most terpenes have pleasant odors, some produce off-odors. A prime example is the musky-smelling sesquiterpenes produced by *Penicillium roquefortii* in cork *(Heimann et al., 1983)*. *Streptomyces* species may also synthesize earthy-smelling sesquiterpenes in cork or cooperage wood.

Terpene compounds in wine are prone to changes during wine production and aging. Some terpenes become cyclic and form lactones, for example, 2-vinyl-2-methyltetrahydrofuran-5-one (from linalool oxides). Other terpenes may transform into ketones, such as a- and b-ionone (aroma of violets), or norisoprenoids such as vitispirane (eucalyptus), β-Damascenone (tropical fruit) and 1,1,6,-trimethyl-1,2-dihydronapthalene (petrol/kerosene aroma) *(Schreier et al., 1976)*. Another example is the conversion of geraniol into three diols, which subsequently can undergo further transformations into hotrienol and nerol oxide. Generally, monoterpene alcohols can easily isomerize and oxidize, forming oxides and aldehydes *(Rapp & Guntert, 1986)*. In addition, the development of *Botrytis cinerea* on grapes may be reduces and modifies their content of terpenes *(Boidron, 1978 & Rapp, 1987)*. This undoubtedly plays a major role in the minimal varietal character of most botrytised wines *(Bock et al., 1988)*.

2.1.4 Acids

Among acids in wine, fatty acids (acetic, formic, butyric, propionic, C_6 to C_{10} carboxylic acids) are the major contributor to the wine fragrance. Their presence in wine is primarily derived from action of yeasts and bacteria. All volatile carboxylic acids have marked odors – acetic acid is vinegary, propionic acid is characterized as fatty, butyric acid resembles rancid butter and C_6 to C_{10} carboxylic acids possess a goaty odor. The acetic acid is the most important because at normal levels in wine (<300 mg/L) it can add complexity to the bouquet *(Ronald S. Jackson, 2008)*. In addition, the major wine acids, notably tartaric and malic, are non-volatile. Lactic acid has a mild odor, but is seldom of sensory significance. These acids may indirectly play a role in wine fragrance by participating in the formation of aromatic esters.

2.1.5 Aldehydes

Aldehydes found in wine may come from grapes, such as C_6 aldehydes (hexanals and hexenals). They appear to be formed during crushing by the enzymatic oxidation of grape linoleic/linolenic acid and may be involved in the grassy to herbaceous odor associated with certain grape varieties, such as 'Grenache' and 'Sauvignon blanc', or with wines made from immature grapes *(Ribıreau-Gayon et al., 2006)*. However, most aldehydes found in wine are produced during fermentation, processing, or extracted from oak cooperage. Acetaldehyde is the major wine aldehyde. It often constitutes more than 90% of its aldehyde content. Above threshold values in table wines, acetaldehyde is considered an off-odor. Combined with other oxidized compounds, it contributes to the traditional bouquet of Sherries and other oxidized wines. Furfural and 5-(hydroxymethyl)-2-furaldehyde are other aldehydes having a vinous sensory impact. Their caramel-like aspects are most evident in baked wines. Phenolic aldehydes, such as cinnamaldehyde and vanillin, may accumulate in wines matured in oak. Other phenolic aldehydes, such as benzaldehyde, may have diverse origins. Its bitter-almond odor is considered characteristic of certain wines, for example, those from Gamay grapes *(Ronald S. Jackson, 2008)*.

2.1.6 Ketones

The complex ketones, β-damascenone and α,β-ionones (isoprenoids) are present, partly as a result of crushing the grapes. The intense exotic flower or rose-like scent of β-damascenone, and its low odor threshold, indicate that it probably plays a contributing role in the aroma of several grape varieties, including 'Chardonnay' *(Simpson & Miller, 1984)* and 'Riesling' *(Strauss et al., 1987)*. The violet/raspberry scent generated by β-ionone, along with β-damascenone, appears to be significant in the aroma of several red grape varieties *(Ferreira et al., 1993)*. Furthermore, many ketones produced during fermentation, but few appear to have sensory significance. The major exception is diacetyl (biacetyl, or 2,3-butanedione). At low concentrations (< 5 mg/l), diacetyl donates a buttery, nutty, or toasty flavor. However, at much above its sensory threshold, diacetyl generates a buttery, lactic off-odor. This commonly occurs in association with spoilage induced by certain strains of lactic acid bacteria *(Rogerson et al., 2001)*.

2.1.7 Acetals

Acetals are formed when an aldehyde reacts with two alcohols and possess vegetal odors. More than 20 acetals have been isolated from wines, which produced during ageing. Their concentration and volatility seem to suggest that they have little sensory impact in table wines *(Montedoro & Bertuccioli, 1986)*.

2.1.8 Lactones

Lactones are cyclic esters formed by internal esterification between carboxyl and hydroxyl groups. They may come from grapes, be synthesized during fermentation and aging, or be extracted from oak barrels. Generally, these compounds have a candy- sweet, coconut, woody, butter and fruity odor *(Guth, 1996)*. More than 20 lactones have been identified in grapes and wines *(Montedoro & Bertuccioli, 1986)*. Among them is:

2-Vinyl-2-methyltetrahydrofuran-5-one, comes from grapes and contributes to aroma of Riesling and Muscat wines *(Schreier & Drawert, 1974)*.

Sotolon (4,5-dimethyl-tetrahydro-2,3-furandione), is a characteristic compound of Botrytis-infected grapes/wine and adds a nutty, candy-sweet, burnt odor *(Masuda et al., 1984)*.

Oak-lactones (isomers of β-methyl-γ-octalactone) known as whisky lactones, come from oak barrels and depending on their chemical structure give different odor characteristics. The trans oak-lactones show spicy and coconut notes, whereas the cis oak-lactones offer notes of fresh wood – oaky, coconut, vanilla and chocolate *(Brown et al., 2006 & Pollnitz P.A. et al., 1999)*.

2.1.9 Nitrogen Compounds

Pyrazines are cyclic nitrogen-containing compounds, are extracted from grape skin during crushing and contribute significantly to the flavor of wines. They are also important to the varietal aroma of several grape cultivars. 2-Methoxy-3 isobutylpyrazine plays the major role in the bell (green) pepper odor often detectable in Cabernet Sauvignon and related cultivars, such as Sauvignon blanc and Cabernet Franc. At concentrations of about 8–20 ng/L, Methoxy-butyl-pyrazines may be desirable, but, at above these values, it starts to generate an overpowering vegetative, herbaceous aroma. Related pyrazines are present but generally occur at concentrations at or below their detection thresholds *(Allen et al., 1996)*.

Pyridines are another group of aromatic cyclic nitrogen compounds periodically isolated from wine. Thus far, their involvement in wine flavor appears to be restricted to the production of mousy off-odors. This odor has been associated with 2-acetyltetrahydropyridines, 2-ethyl-tetrahydropyridine, and 2-acetyl-1-pyrroline *(Heresztyn, 1986 & Grbin et al., 1996)*. They are produced by some strains of Brettanomyces.

2.1.10 Volatile Phenols

Volatile Phenols formed in the fermentation or released in ageing and only few of them come from grapes. Phenols donate a very distinct aroma (pharmaceutical, smoky, tarry, leathery, saddles, manure, sweat, clove, bacon etc.) and contribute positive or negative to odor of wine depending on their concentration (very low odor threshold) *(Liu, 2002)*. An important phenolic flavorant is 2-phenylethanol, which produces the rose fragrance. Another major phenolic alcohol found in wine is tyrosol. This yeasts synthesized phenolic has mild beeswax, honey-like odor.

Two types of phenols are regarded as important, vinylphenols (4-vinylguaiacol and 4-vinylphenol) and ethylphenols (4-ethylphenol and 4-ethylguaiacol). These compounds produced with biotransformation of the hydroxycinnamic esters (notably coumaric and ferulic acids) by *Brettanomyces*. Hydroxycinnamic esters are precursors naturally present in grapes in the bound or free form. Only the free form is used by Brettanomyces *(Chatonnet et al 1997)*. Vinylphenols and ethylphenols donate spicy, pharmaceutical, clove-like odors, and smoky, animal, stable-like notes, respectively. Off-odors are frequently detected when ethylphenols contents exceed 400 mg/liter, or 725 mg/liter for vinylphenols *(Chatonnet et al., 1992)*. Red wines typically show a greater proportion of ethyl to vinyl-phenols, and a higher absolute concentration of these compounds. The reverse is characteristic of white wines *(Ribéreau-Gayon et al., 2006 & Suarez et al., 2007)*.

Eugenol found in wine fermented or matured in oak cooperage and adds a general spicy note. Guaiacol generated from thermal degradation of oak lignins and has a sweet, smoky odor *(Dubois, 1983)*. Additionaly, oak cooperage is the source of several volatile phenolic acids and aldehydes *(Chatonnet et al., 1990)*. For example, benzaldehyde is particularly prominent and possesses an almond-like odor *(Goetghebeur et al., 1992)*. Other important phenolic aldehydes are vanillin and syringaldehyde. Both of them possess vanilla-like fragrances and formed during the breakdown of wood lignins. The toasting of oak staves during barrel construction is another source of volatile phenolic aldehydes, notably furfural and related compounds *(Dourtoglou et al., 1994)*.

2.1.11 Sulfur Compounds

Hydrogen sulfide (H_2S) and sulfur-containing organics generally appear only in trace amounts in bottled wine. Have been found that sulfur compounds in wine confer strong flavors with very low threshold levels *(Charpentier & Mau jean, 1981)*. These compounds classified into five categories, according to their chemical structure: thiols, mercaptans, thioesters, sulfides, and heterocyclic compounds. Most of them impart aroma similar to cabbage, garlic, onion, rubber etc. (table 2). Generally, sulfur compounds degrade the quality of the wine.

Table 2: Odor of some sulfur compounds in wine *(Tominaga & Dubourdieu, 2006 & Tominaga et al., 2000 & Tominaga et al., 2003 & Murat M.L.,2005)*.

Sulfur compounds	Odor
Hydrogen sulfide	Rotten eggs
Methanethiol	rubber / rotten cabbage
2-mercaptoethanol	burnt rubber
ethanedithiol,	rotten onion, burnt rubber
2-Mercaptoethyl acetate 3-mercaptopropyl acetate	Grilled - roasted meat
dimethyl sulfide	cooked cabbage, shrimp
4-Mercapto-4-methylpentan-2-ol	Citrus zest
3-mercaptohexan-1-ol	grapefruit
4-mercapto-4-methylpentan-2-one 3-mercaptohexyl acetate	box-tree
3-methylbutan-1-ol	cooked leeks
p-Menthene-8-thiol	spicy/ tropical
Benzenemethanethiol	Gunflint, smoke
2-Methyl-3-furanthiol	Cooked meat
Ethyl methionate	Mushrooms
2-furanmethanethiol	coffee notes

2.1.12 Hydrocarbons Derivatives

Hydrocarbons are compounds composed solely of carbon and hydrogen atoms. Several hydrocarbons in grapes generate important volatile degradation products, which influence the sensory characteristics of wine. Examples are b-damascenone, a- and b-ionone, and vitispirane. b-Damascenone has a pleasant fruity/floral aroma, vitispirane has an floral, fruity, woody, or reminiscent of eucalyptus fragrance and a- / b-ionone donate a violet odor. Many of them are derived from the hydrolytic degradation of carotenoids *(Sefton et al., 1989)*.

Possibly the most significant aromatic hydrocarbon present in grapes, or subsequently generated in wine, is the 1, 1, 6-trimethyl-1, 2-dihydronaphthalene (TDN). TDN has smoky, kerosene, bottle-aged fragrance and is responsible for the “petrol” or “kerosene” aroma typically found in aged Rieslings *(Winterhalter, 1991)*. The sensory threshold is about 20 ppb and may be desirable at low concentrations *(Simpson, 1978)*.

A cyclic hydrocarbon occasionally found in wine is styrene. It can produce a taint in wine stored in plastic cooperage or transport containers *(Hamatschek, 1982)*. Additional hydrocarbon taints may come from methyl tetrahydronaphthalene, implicated in some types of corky off-odors *(Dubois P. & Rigaud J., 1981).*

2.2 Analysis of Volatile Compounds

Volatile aroma compounds in wines (or grapes) are separated, detected and quantified by gas-chromatography with a mass spectrometry detector (GC/MS). The equipment basically consists of an inlet for the sample, a column on which the sample is separated and a means of detecting the volatile compounds. The important aspect of this equipment is the choice of column. Capillary columns are now regarded as essential and appropriate for an effective separation. A controlled gas flow through the column and a gradually increasing temperature are used to separate the volatile compounds over the column. Gas-chromatography equipment allows the identification and quantification of very low concentrations of compounds *(Dominic M. & Nico M., 2010)*.

The main functional parts of mass spectrometry detector are the ion source and spectral analyzer. As ion source can be the EI method (electron ionization) with electron impact or the CI method with chemical ionization *(Mark & Dunn, 1985 & Harrison, 1983)*. The identification of eluting volatile compounds, done by matching the fragmentation pattern and molecular ion obtained by MS with specialist reference libraries of such information by using a computer *(Dominic M. & Nico M., 2010)*.

A very important part of the analysis is the sample preparation. The sample preparation methods have been much improved both in quality of the sample and ease of preparation. For example, the method of SPE (solid-phase extraction), which used to extract, clean and concentrate samples without using of solvents *(Dominic M. & Nico M., 2010)*. Different methods of solid phase extraction (SPE) of wine volatiles were developed by using Amberlite XAD - 2 polystyrenic resins , reverse - phases C 18 , highly cross - linked hydroxylated polystyrenic phases and highly cross - linked ethylvinylbenzene – divinylbenzene copolymers stationary phases *(Gunata et al., 1985 & Versini et al., 1988 & Williams et al., 1982 & Di Stefano, 1991 & Zulema et al., 2004 & Ferreira et al., 2004 & Boido et al., 2003 & López et al., 2002 & Genovese et al., 2005)*. For extraction of terpenes reversed phase SPE provides is the most effective, whereas LiChrolut EN resins and XAD-2 resins perform more effectively in the extraction of acids, benzenes, phenol *(N. Loscos et al., 2009)*.

The alternative method, which has been widely used, is to extract the volatile compounds by a solvent, followed by distilling and condensing. In the Likens–Nicherson combined extraction–distillation apparatus, pentane–methylene chloride is a popular solvent *(Vernin et al., 1993)*. Other solvents, such as the freons have been described for use with wine volatiles *(Rapp & Marais, 1993)*, along with operation under vacuum to lower the extracting temperature in extraction–distillation *(Grosch, 2001)*.

Chapter 3

Purpose of this Study

Cabernet franc has unique aroma compounds and produces a floral, fruity and sometimes vegetal aromatic character. The type of yeast and lactic acid bacteria used, and the conditions of fermentations process affect the organoleptic qualities of wines *(Waterhouse & Ebeler, 1998)*.

The purpose of this study was to compare four different vinification, connected with different fermentation management practices in order to evaluate possible alternative process to improve the aromatic qualities of the variety Cabernet Franc from the area DOC Colli Orientali.

Chapter 4

Material and Methods

4.1 Wine samples

During harvest 2015, four wines were elaborated from one batch of the grapes variety, Cabernet Franc (DOC Colli Orientali del Friuli, North-East Italy), in 100-L containers. After destemming and crushing two of the musts inoculated with *Saccharomyces cerevisiae* selected yeasts (Anchor NT202, Oenobrands, Montferrier-sur-Lez, France), while the remaining two samples were not inoculated (spontaneous fermentation). The Inoculated yeasts rehydrated with water at temperature of 37°C, and mixed, according to supplier's instructions (starter culture). The starter culture needed 30 minutes to be ready. Then, the temperature of the starter culture is slowly reduced by the graduated addition of must, and after 20 minutes added to must. The fermentation temperature maintained at 18-20°C. After two days from the start of alcoholic fermentation was carried out the inoculation with Lactic Acid Bacteria (self-prepared culture of five different strains of *Oenococcus oeni*) at a concentration of 106 CFU/mL. This process was conducted in one of the samples that had been inoculated with *Saccharomyces cerevisiae* selected yeasts and in one of not inoculated.

The maceration was done for fifteen days and solids were punched down twice a day. Subsequently, each sample drained and pressed separately, and juice transferred in 54-L glass containers. A few days before the end of the fermentation, the wine was decanted into smaller containers in order to protect from oxidation and the lees produced during fermentation. After about 20 days of the end of the alcoholic fermentation was carried out bottling and was added 50 mg/L of sulfur dioxide to each bottle. The fermentations were carried out without the addition of sulfur dioxide.

4.1.1 Characteristics of yeasts and bacteria starter

Anchor NT202 (Oenobrands, Montferrier-sur-Lez, France)

Species	Hybrid of S. cerevisiae
Fermentation kinetics	Fast and normal
Cold tolerance	18 °C
Optimum temperature range	20-28°C
Osmotolerance	26°Brix
Alcohol tolerance (20°C)	16% vol
Foam production	Low
Glycerol production	9-12 g/l
Volatile acidity production	< 0.3 g/l
SO2 production	Low
Nitrogen requirement	Average
Dosage	30 g/hL

Aromatic flavors: red berries, mint, blackberries, black currant, tobacco.

It has a stimulating effect on malolactic fermentation and indicated for high alcoholic musts (high alcohol tolerance) *(http://www.oenobrands.com/en/our-brands /anchor /new-world-wine-yeasts/product-data-sheets/nt-202)*.

Lactic acid bacteria starter (*Oenococcus oeni*)

- not produce biogenic amines
- good tolerance to ethanol
- good resistance to SO_2
- presence different genotypes

This Lactic acid bacteria starter, it was a self-prepared culture of five different strains of *Oenococcus oeni*, previously isolated from the winery where the fermentations were conducted. These specific strains were genetically and physiologically characterized at the laboratories of Food Microbiology of the University of Udine (Dipartimento di Scienze Agroalimentari, Ambientali ed Animali) and made available by kind permission of prof. Lucilla Iacumin.

4.1.2 Characteristics of the wine samples

PC: Alcoholic fermentation and malolactic fermentation were carried out by inoculation with yeast starter culture (Anchor NT202) and a self-prepared *Oenococcus oeni* starter culture, respectively. The malolactic fermentation was started at the beginning of alcoholic fermentation. This wine has alcohol content equal to 12,7 % v/v, sugar residue equal to 5,5 g/L and acetaldehyde content equal to 7,5 mg/L.

P: Alcoholic fermentation was carried out by inoculation with yeast starter culture (Anchor NT202), whereas the malolactic fermentation has taken place naturally, without adding Lactic acid bacteria (spontaneous). This wine has alcohol content equal to 12,5 % v/v, sugar residue equal to 7,4 g/L and acetaldehyde content equal to 6,5 mg/L.

WC: Alcoholic fermentation was carried out naturally, without adding yeast starter culture (spontaneous). Malolactic fermentation taken place after inoculation with a self-prepared *Oenococcus oeni* starter culture, at the beginning of alcoholic fermentation (non-spontaneous). This wine has alcohol content equal to 12,5 % v/v, sugar residue equal to 13,3 g/L and acetaldehyde content equal to 11,1 mg/L.

W: Alcoholic fermentation and malolactic fermentation were carried out naturally, without adding starter cultures. This wine has alcohol content equal to 12,9 % v/v, sugar residue equal to 6,2 g/L and acetaldehyde content equal to 6,2 mg/L.

4.2 Analysis of aromatic compounds

To each wine sample carried out extraction and identification of aromatic compounds (free and bound terpenes, volatile compounds).

4.2.1 Extraction of Free and bound Terpenes

1-Heptanol solution in ethanol (500μg/mL) was used as internal standard. A 100μL portion of this solution was added to 100ml of wine.

An isolute C18 - SPE (500mg RP) column (Biotage, Sweden) was used to capture the aromatic components (Fig.1). Mobilization of the column was performed by adding 25mL of methanol (CH_3OH) and 25mL of Milli-Q water (flow ~ 3mL/min).

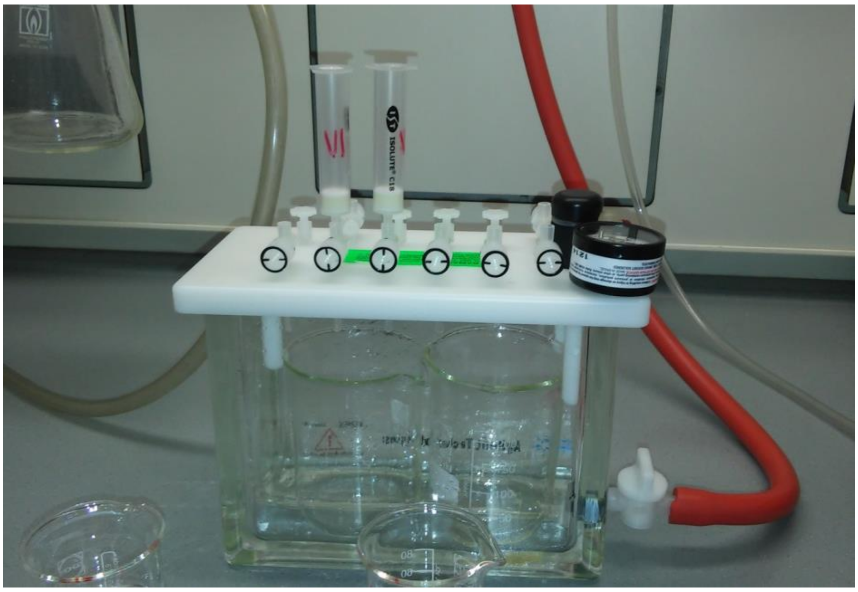


Figure 1: Filter Isolute C18 – SPE.

Wine was passed through the SPE column at a low flow rate about 1 ml/min. After loading of sample onto the column, it was rinsed out with 150mL of Milli-Q water to eliminate sugars and other low-molecular-weight polar compounds (flow ~ 3mL/min).

Free fraction (Non-polar fraction) was eluted (flow ~ 3mL/min) using 25mL of a mixture of pentane: dichloromethane (2:1, v/v). Subsequently, the free fraction concentrated by nitrogen flow to obtain about 8mL of solution, and dried by adding excess of anhydrous sodium sulphate (Na_2SO_4).

The bound fraction (polar fraction) was eluted using 25mL of methanol, and then was evaporated to dryness under nitrogen stream (low flow for 4h) (Fig.2). The fraction rehydrated using 5mL of buffer solution at pH 5 (Mc'Ilvaine citric, 0.2M) according to method of Ferreira *(Ferreira et al., 2006)*. Addition of buffer carried out in three doses of 2mL, 2mL and 1mL using Votrex for good mixing. During the first addition the solution was heated using water bath at 100°C for some minutes.

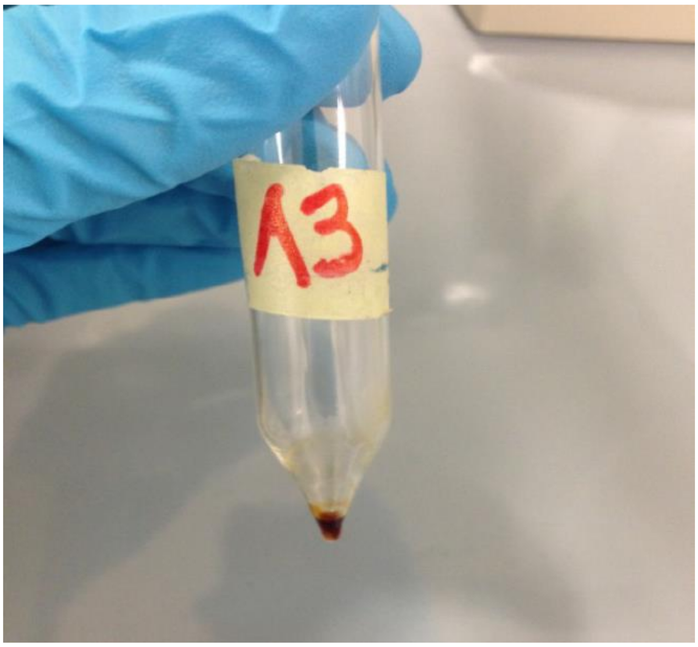


Figure 2: Dried fraction.

After the rehydration was added 200mL of an enzymatic solution with a view to hydrolysis of bound fraction. Hydrolysis carried out by heating in the oven at 40°C for 24h. The used pectolytic enzyme is the alpha and beta glucosidase, and recommended in doses of 1 to 4g/hl. The solution containing this enzyme was prepared by adding 250mg of enzyme in 10mL of Milli-Q water.

After the enzymatic hydrolysis the content of the tube was transferred into 10-mL volumetric flask by washing with buffer solution (10mL). A 100µL portion of 1-heptanol solution in ethanol (500µg/mL) was added as an internal standard, and the sample was transferred into a testing tube.

The mixture was then extracted three times with 15mL of pentane: dichloromethane (2:1, v/v). The first extraction was performed by a vigorous shaking of tube for 2 minutes, after addition of 5mL NaCl and 5mL 5-DCM. With the shaking are created two phases, the organic on the top and the water phase on the bottom (Fig.3). After the transfer of organic phase, were added other 5 mL of 5-DCM and the mixture was shaken for two more minutes. The new organic phase was removed and added to the first one. Subsequently, was done the same action with addition of 5 mL 5-DCM one more time.

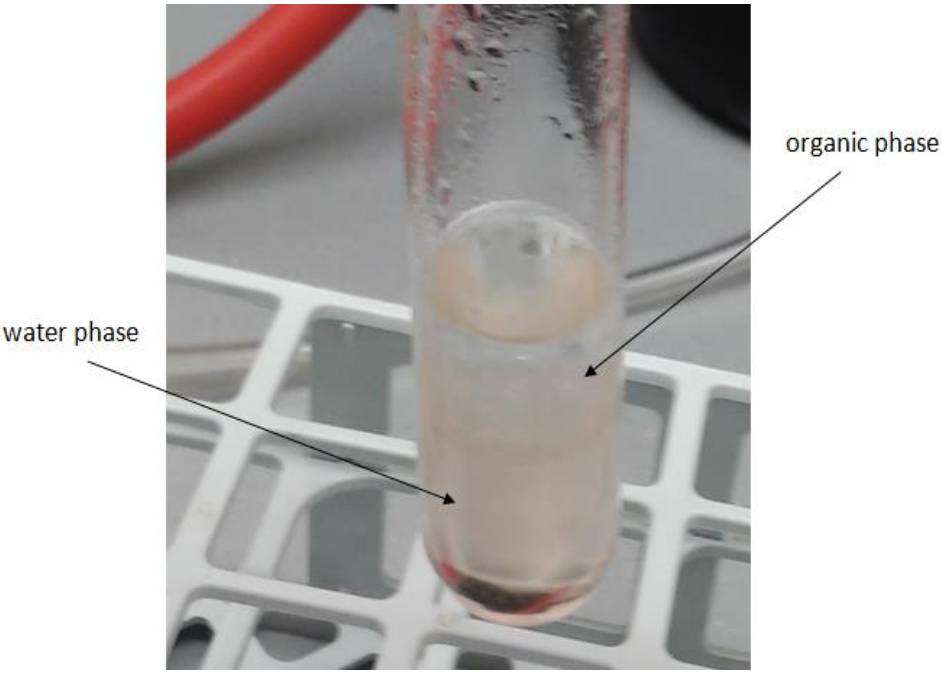


Figure 3: Extraction of bound terpenes by addition of 5-DCM.

The sample was concentrated by nitrogen low flow. Nitrogen action gave a water phase which was removed. At the end, solution was dried by adding excess of anhydrous sodium sulphate (Na_2SO_4).

4.2.2 Extraction of volatile compounds

A 200μL portion of ethyl heptanoate solution (~ 0.5 g/L in ethanol) added to 5ml of wine contained into a testing tube. The ethyl heptanoate solution was used as internal standard.

The mixture was then extracted three times with 12.5mL of 5-DCM (2:1, v/v). The first extraction was performed by a vigorous shaking of tube for 2 minutes, after addition of 5mL NaCl 30% and 2.5mL 5-DCM. With the shaking were created three phases. The two phases on the top were the organic fraction, whereas on the bottom was the phase of water (Fig.4). After the transfer of organic phases, were added other 5mL of 5-DCM and the mixture was shaken for two more minutes. The new organic phases were removed and added to the solution which containing the organic phases from the first extraction. Subsequently, was done the same action with addition of 5mL 5-DCM one more time.

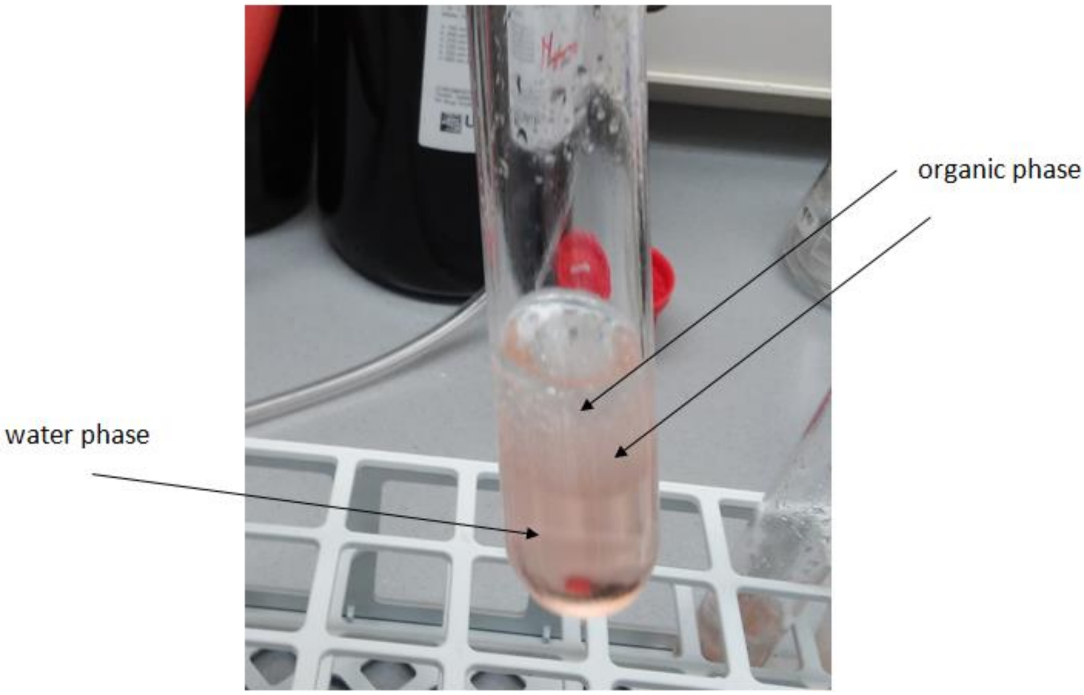


Figure 4: Extraction of volatile compounds using 5-DCM.

The organic fraction was concentrated by nitrogen low flow. Nitrogen action gave a water phase which was removed. At the end, solution was dried by adding excess of anhydrous sodium sulphate (Na_2SO_4).

4.2.3 GC/MS analysis

The extraction solutions were concentrated to 1mL by a low stream of nitrogen. One microliter (1μL) of each solution was injected to a Gas Chromatograph model GC-17A (Shimadzu, Kyoto, Japan).

The Gas Chromatograph was coupled to a Mass Selective Detector model QP 5000 (Shimadzu, Kyoto, Japan) and equipped with a J&W DB-Wax capillary column

(30m - 0.25mm i.d., 0.25µm film thickness), purchased from Agilent Technologies Inc. (Santa Clara, CA, USA).

In case of analysis free and bound terpenes, the injection was done in splitless mode, while for analysis of volatile compounds the injection was done in split - splitless mode. The temperature of injector and detector was set at 250°C. The carrier gas was helium at a linear flow rate of 23 cm/s and constant pressure of 49 kPa. The mass spectrometer was operated in electron ionization mode (EI, 70 eV), with an ion source temperature at 200°C. The column temperature was programmed as follows: 40°C for 5 min, then at 4°C/min up to 240°C, with a final holding time of 15 min *(Comuzzo et al.2006)*.

The identification of compounds was carried out by comparison of their mass spectra and retention times with those of standard compounds, or by comparison of mass spectrum, with those reported in the mass spectrum library Wiley 6, NIST 107 and NIST 21.

Retention indices (RIs) were calculated in accordance with a modified Kovats method. A standard mixture of n-alkanes (C_7-C_{30}) was prepared and injected into the GC. The retention times and the literatures were used to calculate RIs and RILs, respectively *(Cates & Meloan, 1963)*.

The absolute areas of the identified compounds were compared with those of the internal standard in order to determine their concentration in µg/l (Quantitative analysis).

4.3 Sensory analysis

A sensory analysis was conducted by a panel of 12 judges. Panelists were staff members and postgraduate students from the University of Udine (Università degli Studi di Udine). They had experience with the different expressions of wines. The wine samples were poured at room temperature (about 19 ◦C) into ISO tasting glasses *(ISO 3591, 1997)* and coded with random three-digit numbers (Fig.5).

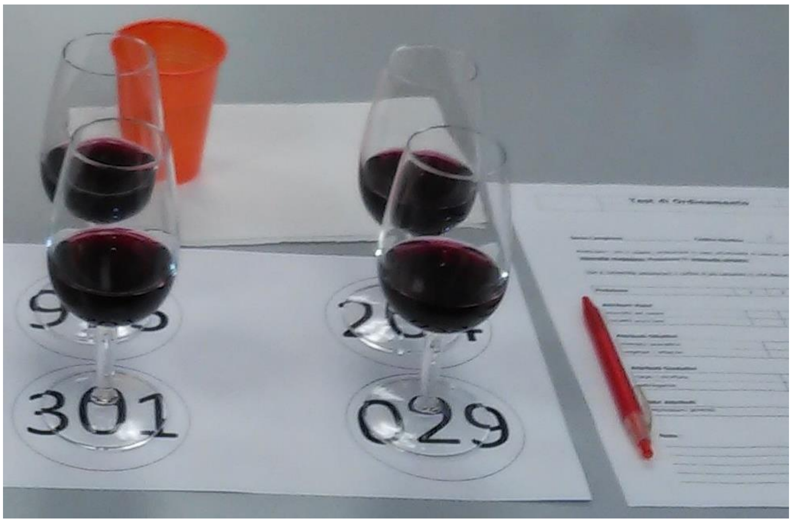
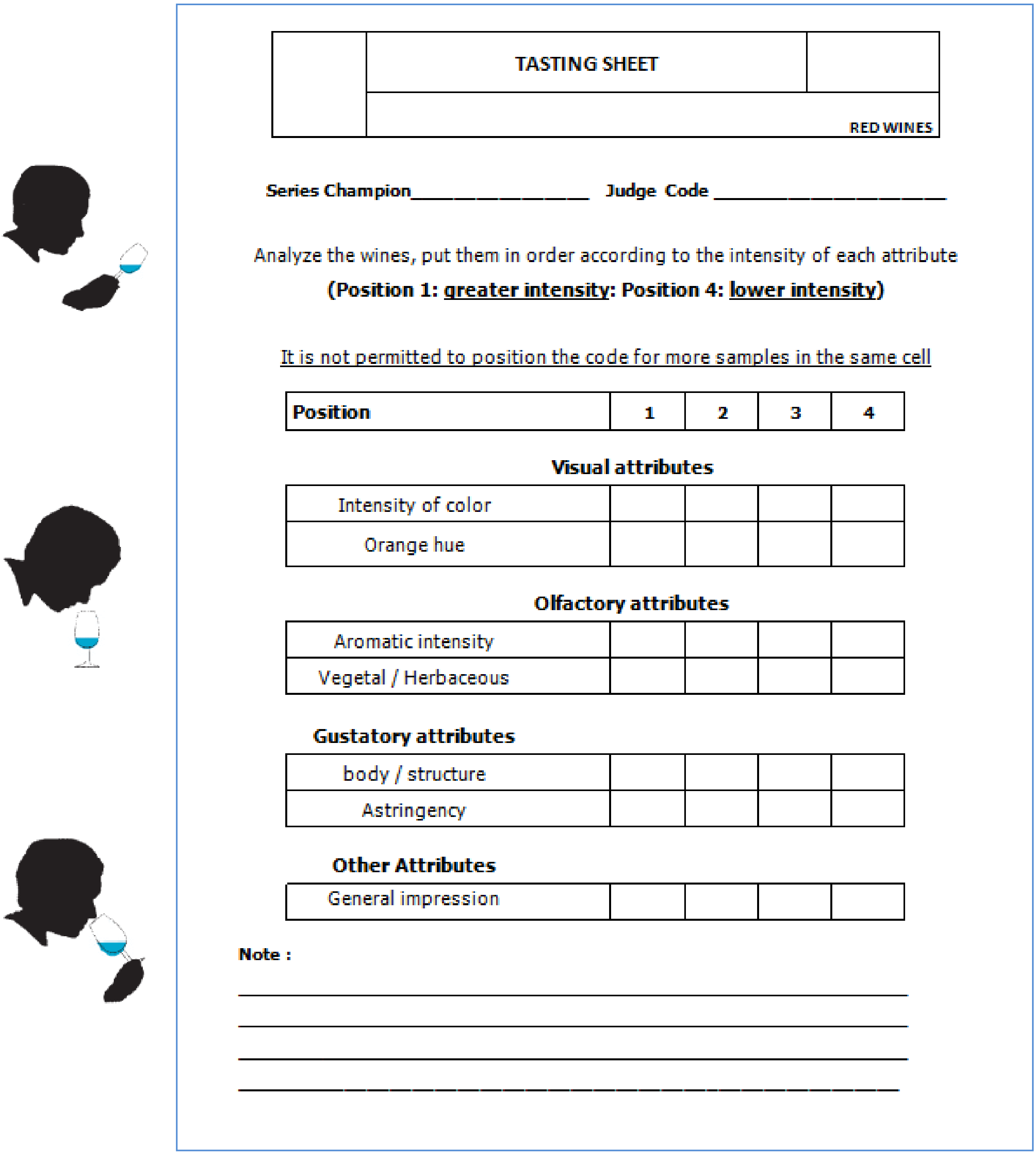


Figure 5: Sensory analysis.

Wines were sniffed and tasted. Judges put them in order according to the intensity of each attribute. The descriptors chosen were intensity of color and orange hue, aromatic intensity and vegetal/ herbaceous aromas, body/ structure and astringency, and general impressions. At Figure 6 is presented the tasting sheet which was used.

The statistical treatment of results was conducted using the Friedman test *(Barillere & Benard, 1986)*, in order to identify the minimum significant difference between the ranks ($p < 0.05$). If there were significant differences between the sums of the ranks, samples for which a given attribute was perceived as more intense, were those with the lowest sum of the ranks.

$$F = \frac{12(R1^2\ R2^2 + ..Rk^2)}{nk(k+1)} - 3n\,(k+1)$$



TASTING SHEET

RED WINES

Series Champion_______________ **Judge Code** ____________________

Analyze the wines, put them in order according to the intensity of each attribute

(Position 1: <u>greater intensity</u>: Position 4: <u>lower intensity</u>)

<u>It is not permitted to position the code for more samples in the same cell</u>

Position	**1**	**2**	**3**	**4**
Visual attributes				
Intensity of color				
Orange hue				
Olfactory attributes				
Aromatic intensity				
Vegetal / Herbaceous				
Gustatory attributes				
body / structure				
Astringency				
Other Attributes				
General impression				

Note :

____

____

____

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Figure 6: Ordinary test.

4.4 Statistical analysis

Significant differences were assessed with one-way analysis of variance (ANOVA), statistical differences between the means were evaluated using Tukey's test at the p = 0.05 level and the contribution of volatile compounds to the wine aroma was estimated by correlation method. Statistical data processing was performed using the Statsoft Statistics 8 MR-3.

Chapter 5

Results and Discussion

5.1 Identification of Terpenes contained to each wine

At the following table presents the terpenes compounds which used in the identification method for the peaks present on the diagrams obtained from the GC/MS analysis.

Table 1: Used compounds in the identification method. RI: Kovat's calculated Indexes, RIL: Retention Indexes from bibliography, IM: Identification method: MS comparison with the mass spectrums present in the bibliography, S comparison with commercial standards. Bibliography: (Choi et al., 2003 & Selli at al., 2004 & Davies, 1990 & http:// www. flavornet. org /dodors.html).

Compounds	RI	RIL	IM	References
Limonene	1194	1187	MS,IR,S	Davies, 1990
a-copaene	1485	1488	MS,IR	Flavornet
Linalool	1556	1506	MS,RI,S	Davies, 1990
a-bergamotene	1585	1590	MS,RI	Davies, 1990
terpinene-4-ol	1601	1601	MS,RI	Davies, 1990
β-citronellol	1765	1722	MS,RI,S	Davies, 1990
Geraniol	1854	1842	MS,RI,S	Davies, 1990
caryophyllene oxide	1971	2000	MS,RI	Davies, 1990
Farnesol	2362	2371	MS,RI	Choi et al., 2003
3-oxo-a-ionol	2619	2651	MS,RI	Selli at al., 2004

5.1.1 Identified terpenes compounds in free form

Table 2 shows the average of concentrations obtained for each compound and expressed as μg/L. a-Copaene was not detected in any of the wines. The most compounds were detected to the WC, including terpinen-4-ol and caryophyllene oxide, which were not presented to the other three wines. Only two terpenes in free form detected to the P wine, β-citronellol and geraniol. Almost the same compounds detected to the W and PC wines, except of a-bergamotene, which presented only to the W wine. Table 2 also includes the analysis of variance (ANOVA) and the Tukey test.

Table 2: Average concentrations (μg/L) of free terpene compounds. Results of ANOVA and Tukey's test. nd: not detected, a b c d : with the same letter do not differ significantly by Tukey's test, $p<0.05$.

compounds	W	WC	P	PC
Limonene	3,79 ± 0,010 c	8,15 ± 0,050 a	nd	6,43 ± 0,025 b
a-copaene	nd	nd	nd	nd
Linalool	0,91 ± 0,005 c	2,54 ± 0,050 a	nd	1,40 ± 0,005 b
a-bergamotene	1,05 ± 0,050 b	2,05 ± 0,050 a	nd	nd
terpinene-4-ol	nd	2,04 ± 0,004	nd	nd
β-citronellol	24,75 ± 0,050 a	21,49 ± 0,010 b	12,85 ± 0,050 d	19,05 ± 0,050 c
Geraniol	41,79 ± 0,015 a	31,00 ± 0,005 b	16,52 ± 0,020 c	9,01 ± 0,010 d
caryophyllene oxide	nd	38,05 ± 0,050	nd	nd
Farnesol	30,05 ± 0,050 a	27,25 ± 0,050 c	nd	29,60 ± 0,000 b
3-oxo-a-ionol	1,35 ± 0,050 c	6,41 ± 0,010 b	nd	14,61 ± 0,010 a
Total	103,69	138,98	29,37	80,1

As for the total concentrations of the free terpene 103,69 μg/L, 138,98 μg/L, 29,37 μg/L and 80,1 μg/L was detected in W, WC, P and PC wines, respectively. All identified terpenes were in low concentrations. As table 2 shows there are significant differences in concentration of identified terpenes between all wines.

Many research showed that terpenes increase following MLF. For example, at a compare of wines produced by Cabernet franc grapes, in which MLF is carried out in steel tanks and then aged in oak barrels, with wines in which MLF has been carried out in barrels, researchers noticed that concentration of citronello nearly doubles. In particular, the concentration from 27,62 μg/L increased to 49,11 μg/L at the wine in steel tanks and to 42,84 μg/L at the wine in barrels *(P.M. Izquierdo-Canas et al., 2015)*. The MLF was carried out after inoculation with a commercial Alpha strain (MBR) at 1 g/HL. Other terpenes identified at the research mentioned above were 3-oxo-a-ionol (57,05 μg/L), linalool (5,42 μg/L) and geraniol (2,27 μg/L). 3-Oxo-a-ionol is an oxygenated C_{13}-norisoprenoids and had also been identified in Tannat wines of the southern part of Uruguay *(E. Boido et al., 2003)*.

At this study, 3-Oxo-a-ionol was presented to W, WC and PC wines, with highest concentration at PC (14,61 μg/L). By comparison of W and WC, it is clear that concentration of 3-oxo-a-ionol is higher when malolactic fermentation carried out by inoculation at the beginning of alcoholic fermentation (WC) than when was carried out naturally (W). In addition, between of WC and PC the 3-oxo-a-ionol was higher in case of non-spontaneous alcoholic fermentation. Norisoprenoids impart fruity, floral or spicy notes. Hence, their presence has very beneficial effects for the sensorial quality of wines.

Citronellol has green lemon, clove and rosebush smells *(Li, 2006, Mateo & Jimenez, 2000)*. The least concentration of β-Citronellol (12,85 μg/L) was observed in P wine and the highest at W (24, 75 μg/L).). By comparison of W to WC and P to PC, was found that β-Citronellol was in higher concentration in cases of non-spontaneous MLF.

Linalool is one of the most important monoterpene with odour threshold 50 μg/L *(Etievant, 1991)*. In this study, the levels of linalool are low in all wines but WC and PC (non-spontaneous malolactic fermentation) samples presenting higher concentration than W (spontaneous malolactic fermentation). Between W and WC, the non-spontaneous malolactic fermentation at the beginning of alcoholic fermentation produced more linalool. Linalool has a characteristic floral aroma with spicy tones and lemon aroma. One more indentified monoterpene is terpinene-4-ol. In the case of Koshu, an indigenous Japanese variety, terpinene-4-ol was identified as the domination monoterpene compound and its odor unit was close to 1 in the must and wine of 'Koshu' grape *(Shimizu & Watanabe, 1981)*. In this work terpinen-4-ol presented only at WC wine in concentration of 2,04 μg/L.

Limonene and Geraniol are also two important monoterpenes. Limonene has floral, green and citrus aroma. Limonene has been detected in Cabernet Gernischt (124,3 μg/L) and Chardonnay wines (36,1 μg/L) *(Bao Jiang & Zhenwen Zhang, 2010)*. WC wine produces the highest concentration of limonene. Geraniol has aroma of roses, citrus-fruit and geranium. Its odour threshold is 30 μg/L *(Guth, 1997)*. W and WC wines include geraniol in higher concentration than its odour threshold. On the other hand P and PC have lower concentration of geraniol. By comparison of W to WC and P to PC, was found that concentration of geraniol reduced in cases of non-spontaneous MLF.

A wide range of functional sesquiterpenes and related compounds are biosynthesized in grapes (and by yeast to some extent) in cultivars such as cabernet Sauvignon, Shiraz and Riesling. Farnesol is one of sesquiterpenoids which have been identified in wine. For example, [E,E]-farnesol (18 mg/L) was one of the five terpenes which were detected in young Cabernet Sauvignon red wine from Changli County (China) *(Yongsheng Tao et al. 2008)*. Also, farnesol has identified in wines of Merlot and Cabernet Sauvignon with concentration of 3601,14 μg/L and 1528,39 μg/L, respectively *(Changzheng Song et al., 2015)*. Farnesol has a convallaria majalis smell. Concentration of farnesol increases during malolactic fermentation or hydrolysis of grape glycosidic extracts *(Andrew L. et al., 2016)*. The organoleptic role of sesquiterpenes in wine has not been clearly established *(Schreier et al., 1976 & Bayonove, 1993)*.

In addition, an investigation in grape berries during ripening at several cultivars of Vitis vinifera (Gewürztraminer, Riesling, Yellow Muscat, Shiraz, Lemberger, Cabernet Sauvignon) showed that Gewürztraminer possessed remarkably high amounts of (E,E)-α-farnesene, (3E,6Z)-α-farnesene and (E,E)-methyl farnesoate, while the sesquiterpene profiles of Riesling, Lemberger, Shiraz and Yellow Muscat were dominated by bi- and polycyclic sesquiterpenes. In addition, cabernet Sauvignon showed only minor profile changes during ripening and its comparatively low sesquiterpene hydrocarbon amounts were dominated by (E)-β-caryophyllene, α-humulene and an unidentified sesquiterpene *(Bianca May & Matthias Wust, 2011)*.

Between the four wines, farnesol was detected in the free fraction of W (30,05 μg/L), WC (27,25 μg/L) and PC (29,60 μg/L) wines. W wine had the higher amount of farnesol, which reduced at WC wine, in which malolactic fermentation carried out by inoculation at the beginning of alcoholic fermentation. PC wine produced less farnesol than W but in high concentration.

a-Bergamotene was one of the sesquiterpenes synthesized by all microbial strains (*Penicillium, Trichoderma, Aspergillus, Mucor, Monilia, Streptomyces and Botrytis cinerea*) at a correlation between microbial growth and formation of volatile components in the wine-stopper cork *(Niessen,2001)*. W and WC wines presented this sesquiterpene. Probably, during naturally alcoholic fermentation develop microbial strains which produce this compound. Also, caryophyllene oxide is one more sesquiterpene presented at WC wine and this is the first time that identify in wine.

It is clear that should be done further analyses around these identified sesquiterpenes because there is not much information about their presence in wine.

5.1.2 Identified terpene compounds in bound form

Table 3 shows the average of concentrations obtained for each compound and expressed as μg/L. The most bound compounds were detected to the W wine, including a-copaene, caryophyllene, caryophyllene oxide and 3-oxo-a-ionol. a-Copaene was not presented to the other three wines. Only three terpenes in bound form detected to the P, W and PC wines but not the same compounds. Limonene included to the P and PC wines, caryophyllene presented to WC and P, caryophyllene oxide presented to WC and 3-oxo-a-ionol included to all of them. Table 3 also includes the analysis of variance (ANOVA) and the Tukey test.

Table 3: Average concentrations (μg/L) of bound terpene compounds. Results of ANOVA and Tukey's test. nd: not detected, a b c d : with the same letter do not differ significantly by Tukey's test, p<0.05 .

Compounds	W	WC	P	PC
limonene	nd	nd	22,84 ± 0,035 a	9,33 ± 0,025 b
a-copaene	4,18 ± 0,020	nd	nd	nd
linalool	nd	nd	nd	nd
a-bergamotene	nd	nd	nd	nd
terpinene-4-ol	nd	nd	nd	nd
β-citronellol	nd	nd	nd	3,77 ± 0,035
geraniol	nd	nd	nd	nd
caryophyllene oxide	136,60 ± 0,000 a	11,04 ± 0,040 b	nd	nd
farnesol	nd	nd	nd	nd
3-oxo-a-ionol	28,76 ± 0,040 c	21,91 ± 0,010 d	455,71 ± 0,010 a	51,71 ± 0,010 b
Total	169,54	32.95	478,55	64,81

The wines studied contained more but in lower concentration terpenes in free form than in bound form. Terpinene-4-ol, farnesol, linalool, a-bergamotene and geraniol were presented only to the free fraction of wines. a-Copaene was detected only in the bound fraction of W wine. The presence of this compound requires further analysis because is the first time that it identify in a wine.

The bound compounds, do not contribute directly to the aroma, but are a reservoir of odourless precursors of flavour. Enzymatic or acid hydrolysis releases the free forms and increases the aroma *(Cabaroglu et al., 2002; Strauss et al., 1986)*.

5.2 Identification of Volatile compounds contained to each wine

At the following table presents the volatile compounds which used in the identification method for the peaks present on the diagrams obtained from the GC/MS analysis. These compounds are higher alcohols, ethyl esters, fatty acids, lactones and Sulfur compounds, and have previously reported to many studies as fermentation derivatives *(Ames & Elmore, 1992 & Ames & McLeod, 1985 & Munch et al., 1997 & Munch & Schieberle, 1998)*. General, the aroma compounds produced during fermentation include alcohols, esters, acids, and carbonyl compounds *(Robinson et al., 2014a)*. Carbonyl compounds and acids have minimal effects on sensory aspects of wines (except acetaldehyde, diacetyl and acetic acid), while alcohols, esters, terpenes, norisoprenoids contribute substantially *(Robinson et al., 2014a)*.

Table 4: Used compounds in the identification method. RI: Kovat's calculated Indexes, RIL: Retention Indexes from bibliography, IM: Identification method: MS comparison with the mass spectrums present in the bibliography, S comparison with commercial standards. Bibliography: (Lopez et al., 1999 & Varming et al., 2004 & Baek & Cadwallader, 1999 & Jennings & Shibamoto, 1981 & Selli et al., 2004 & Munch et al., 1997 & Wong & Bernhard, 1988 & Franco M.R.B. & Shibamoto T. 2000 & Madruga & Mottram, 1998 & Boulanger R. & Crouzet J. 2000 & http:// www. flavornet. org /d_odors.html).

Compounds	RI	RIL	IM	References
2-methyl-1-propanol	1091	1096	MS,IR	Lopez et al., 1999
2- and 3-methyl-1-butanol	1208	1209	MS,IR,S	Baek & Cadwallader, 1999
ethyl hexanoate	1235	1234	MS,IR,S	Baek & Cadwallader, 1999
ethyl lactate	1340	1358	MS,IR,S	Flavornet
1-hexanol	1356	1359	MS,IR,S	Lopez et al., 1999
cis-3-hexen-1-ol	1384	1387	MS,IR	Baek & Cadwallader, 1999
ethyl octanoate	1434	1435	MS,IR,S	Baek & Cadwallader, 1999
acetic acid	1443	1451	MS,IR,S	Baek & Cadwallader, 1999

acetic acid	1491	1490	MS,IR	Madruga & Mottram, 1998
ethyl 3-hydroxybutanoate	1513	1483	MS,IR	Boulanger R., & Crouzet J. 2000.
2,3-butanediol	1540	1545	MS,IR	Baek & Cadwallader, 1999
2-methylpropanoic acid	1566	1548	MS,IR,S	Munch et al., 1997
1,3-butanediol	1577	1566	MS,IR	Franco M.R.B. & Shibamoto T. 2000
1,2-propanediol	1587	1594	MS,IR	Wong & Bernhard, 1988
diidro-2(3*H*)-furanone (g -butyrolactone)	1608	1632	MS,IR,S	Jennings & Shibamoto, 1981
butanoic acid	1626	1612	MS,IR,S	Munch et al., 1997
ethyl decanoate	1639	1634	MS,IR,S	Lopez et al., 1999
3-methylbutanoic acid	1669	1672	MS,IR,S	Baek & Cadwallader, 1999
diethyl succinate	1673	1642	MS,IR	Jennings & Shibamoto, 1981
3-(methylthio)-1-propanol (methionol)	1710	1714	MS,IR	Lopez et al., 1999
ethyl 4-hydroxybutanoate	1800	1819	MS,IR	Selli et al., 2004
2-phenethyl acetate	1800	1820	MS,IR	Baek & Cadwallader, 1999
hexanoic acid	1844	1852	MS,IR,S	Baek & Cadwallader, 1999
2-phenylethanol	1900	1922	MS,IR,S	Baek & Cadwallader, 1999
octanoic acid	2060	2060	MS,IR,S	Lopez et al., 1999
4-vinylguaiacol	2181	2181	MS,IR	Varming et al.,2004
ethyl hexadecanoate	2254	2250	MS,IR	Flavornet
decanoic acid	2254	2229	MS	Lopez et al., 1999

5.2.1 Qualitative and quantitative analyses of volatile compounds

Cabernet franc has unique aroma compounds common to cultivars typical of the Bordeaux region. Their wines have substantial concentrations of 2-furanmethanethiol; other relevant compounds in Bordeaux cultivars include several thiol compounds (e.g. 2-mercaptoethyl acetate, 3-mercaptohexanol, 3-mercapto-2 methylpropanol) *(Tominaga, Blanchard, Darriet, & Dubourdieu, 2000)*. Cabernet franc is also known for its green pepper characteristic attributable to 2-methoxy-3-isobutylpyrazine *(Roujou de Boubee, van Leeuwen, & Dubourdieu, 2000)*. 4-Ethylphenol and 4-ethyl guaiacol are also specific to Bordeaux cultivars *(Rayne & Eggers, 2008)*.

A report describing impacts of yeast strain on aroma compounds in icewines produced by Cabernet franc grapes (Ontario) showed that spontaneous wines had the most compounds quantified, were the only ones containing geranyl acetone and ethyl benzoate and produced the highest concentration of 1-hexanol, phenylethanol, citronellol, nerol, b-phenyl acetate, and γ-nonalactone *(Kinga Synos et al., 2015)*.

Previous research in Tempranillo wine samples indicated that the concentration of many volatile compounds increases after completion of a spontaneous malolactic fermentation. Specifically, increase occurred in the content of succinic acid esters (ethyl monosuccinate, diethyl succinate and ethyl-methyl succinate), lactones (b ethoxy-gbutirolactone and b-(1-hydroxy-ethyl)-g-butirolactone), terpenes (a terpineol), norisoprenoids (damascenone and 3-hydroxy-b-damascenone) and volatile phenols (vanillin and siringaldehide) *(P.M. Izquierdo Canas, et al. 2008)*.

Table 5 shows the average of concentrations obtained for each volatile compound and expressed as µg/L. Totally, were identified and quantified 26 compounds including higher alcohols, ethyl esters, fatty acids, lactones and sulfur compounds. PC sample presents all of the volatile compounds that were used in the identification method. Butanoic acid and ethyl decanoate were not detected in any of the wines. Table 5 also includes the analysis of variance (ANOVA) and the Tukey test.

Table 5: Average concentrations (µg/L) of volatile compounds. Results of ANOVA and Tukey's test. nd: not detected, a b c d : with the same letter do not differ significantly by Tukey's test, $p<0.05$.

	Compounds	**W**	**WC**	**P**	**PC**
Ethyl esters	Diethyl succinate	0,29 ± 0,010 c	0,29± 0,015 c	0,69 ± 0,015 a	0,39± 0,010 b
	Ethyl decanoate	nd	nd	nd	nd
	Ethyl hexanoate	0,20 ± 0,005 a	0,09± 0,010 b	0,09 ± 0,010 b	0,20 ± 0,005 a
	Ethyl hexadecanoate	0,38 ± 0,020 b	0,30 ± 0,000 c	0,19 ± 0,010 d	0,60 ± 0,005 a
	Ethyl 3-Hydroxybutanoate	0,19 ± 0,010 a	nd	0,1 ± 0,005 b	0,09 ± 0,008 b
	Ethyl 4-hydroxybutanoate	nd	5,32± 0,020 b	4,80± 0,005 c	5,97± 0,030 a
	Ethyl lactate	28,20 ± 0,005 a	1,02 ± 0,015 c	1,89 ± 0,015 b	1,09 ± 0,015 c
	Ethyl octanoate	0,20 ± 0,000 a	0,1 ± 0,005 b	0,1 ± 0,005 b	0,19 ± 0,010 a
	2-Phenethyl acetate	0,19± 0,010 b	0,19 ± 0,015 b	0,1 ± 0,005 c	0,29 ± 0,015 a
	Subtotal	29,65	7,31	7,96	8,82
	Subtotal (%)	7,3	2,1	2,6	2,7
Higher alcohols	2- and 3-methyl-1-butanol	202,69 ± 0,015 a	161,20 ± 0,005 c	151,89± 0,010 d	199,60 ± 0,005 b
	1,3-Butanediol	3,98 ± 0,025 b	4,49 ± 0,015 a	3,08 ± 0,020 c	2,98 ± 0,020 c
	2,3-Butanediol	9,19 ± 0,015 a	8,89± 0,015 b	7,39 ± 0,010 c	6,88 ± 0,025 d
	cis-3-hexen-1-ol	nd	nd	0,09± 0,010 a	0,09 ± 0,015 a
	2-Ethyl-1-hexanol	nd	nd	nd	0,09 ± 0,010
	1-Hexanol	1,79 ± 0,015 a	1,68 ± 0,025 b	1,61 ± 0,005 b	1,60 ± 0,005 b

	2-Methyl-1-propanol	49,61 ± 0,010 a	47,11 ± 0,010 b	38,72 ± 0,020 c	9,10 ± 0,005 d
	2-Phenylethanol	83,01 ± 0,015 b	78,50 ± 0,005 c	69,01± 0,005 d	95,48± 0,020 a
	1,2-Propanediol	nd	nd	nd	0,1 ± 0,005
	Subtotal	350,27	301,87	271,79	315,92
	Subtotal (%)	85,9	88,5	90,0	95,9
Volatile phenols	4-Vinyl guaiacol	nd	nd	nd	0,09 ± 0,010
	Subtotal	-	-	-	0,09
	Subtotal (%)	-	-	-	0,0
Fatty acids	Acetic acid	21,02 ± 0,020 b	25,43 ± 0,025 a	16,81 ± 0,010 c	nd
	Butanoic acid	nd	nd	nd	nd
	Decanoic acid	0,29 ± 0,015 b	nd	0,39 ± 0,015 a	0,39 ± 0,010 a
	Hexanoic acid	1,01 ± 0,005 b	0,89± 0,015 c	0,80 ± 0,005 d	1,18 ± 0,020 a
	3-Methylbutanoic acid	0,39 ± 0,010 c	0,40 ± 0,005 c	0,50 ± 0,005 b	0,69 ± 0,015 a
	2-Methylpropanoic acid	0,79 ± 0,015 d	1,29 ± 0,015 a	1,20 ± 0,005 b	1,03 ± 0,025 c
	Octanoic acid	1,01 ± 0,005 a	0,79± 0,015 b	0,19 ± 0,010 d	0,39 ± 0,015 c
	Subtotal	24,51	28,8	19,89	3,68
	Subtotal (%)	6,0	8,4	6,6	1,1
Lactones	Diidro-2(3*H*)-furanone (g -butyrolactone)	1,69 ± 0,010 b	2,60 ± 0,005 a	1,58 ± 0,020 c	0,19 ± 0,010 d
	Subtotal	1,69	2,60	1,58	0,19
	Subtotal (%)	0,4	0,8	0,5	0,0
Sulfur compounds	3-(methylthio)-1-ropanol (methionol)	1,78± 0,020 a	0,60 ± 0,005 c	0,89± 0,015 b	0,89 ± 0,010 b
	Subtotal	1,78	0,6	0,89	0,89
	Subtotal (%)	0,4	0,2	0,3	0,3
Total		407,9	341,18	302,11	329,59

As for the total concentrations of the volatile compounds, 407,9 µg/L, 341,18 µg/L, 302,11 µg/L and 329,59 µg/L was detected in W, WC, P and PC wines, respectively. P wine had the lowest amount of volatile compounds. The wines which were produced by spontaneous alcoholic fermentation (W, WC) had the highest amount of volatile compounds, especially W wine which produced by spontaneous alcoholic and malolactic fermentation. Moreover, PC sample in which malolactic fermentation were carried out by inoculation with yeast starter culture (Anchor NT202) at the beginning of alcoholic fermentation, had higher amount of aroma compounds than P in which malolactic fermentation were carried out naturally.

All identified ethyl esters were in low concentrations. The highest concentration of ethyl esters are presented at the W sample. The same ethyl esters but in different subtotal concentration were identified at P and PC wines. Actually, at PC wine many of identified ethyl esters are presented at higher amount than at P sample. By comparison of W and WC wines, Ethyl 3-hydroxybutanoate was identified when malolactic fermentation carried out naturally (W) and Ethyl 4-hydroxybutanoate when carried out by inoculation at the beginning of alcoholic fermentation (WC). Ethyl lactate is the most important ester typically playing a role in MLF *(Ruiz-Perez et al. 2012, Ugliano & Moio, 2005)*, resulting from esterification of the lactate produced by lactic acid bacteria (LAB) and the ethanol produced during alcoholic fermentation (AF). Ethyl lactate concentrations rise significantly following MLF, and some authors *(Ruiz-Perez et al. 2012)* have reported that concentrations reached are dependent on the strain of Oenococcus oeni used. It gives wines milky notes and is responsible for the volume feeling *(Ugliano & Moio, 2005)*. Results from this study show similar concentrations of ethyl lactate in wines (WC, PC) in which MLF was carried out after inoculation with a self-prepared Oenococcus oeni starter culture, at the beginning of alcoholic fermentation (non-spontaneous). On the other hand, at the wines in which MLF carried out naturally, without adding Lactic acid bacteria (spontaneous) ethyl lactate occurs at higher concentrations, especially at W sample (28,20 μg/L).

Ethyl esters of fatty acids and acetates have long been considered important contributors to wine aroma, because they occur in wines as major volatile constituents and because they exhibit fruity odors similar to those often used to describe wines *(Etiévant, 1991)*. For example, diethyl succinate contributes to the aroma of wines, supplying fruit and melon notes to the wine and has on odor threshold of 1.2 mg/*L* *(Peinado et al. 2004)*.

As shown in Table 5, higher alcohols were the largest group in terms of the concentration of aroma compounds. W wine had the highest concentration of higher alcohols while P had the less. The most abundant and common alcohol in the four wines was 2- and 3-methyl-1-butanol. By comparison of W and WC wines, the malolactic fermentation were carried out by inoculation at the beginning of alcoholic fermentation decreased the subtotal concentration of alcohols especially the concentration of 2- and 3-methyl-1-butanol. In addition, PC wine has the most amount of higher alcohols, including 2-ethyl-1-hexanol and 1,2-propanediol, which were not detected to the other three wines. By comparison of P and PC wines, the malolactic fermentation when carried out by inoculation at the beginning of alcoholic fermentation increased the amount and subtotal concentration of alcohols. For example, 2- and 3-methyl-1-butanol and 2-Phenylethanol were increased. W, WC and P wines had higher concentration of 2-Methyl-1-propanol than PC, with significant differences between them. Also, W, WC and PC had the same higher alcohols except of cis-3-hexen-1-ol, which indentified at P and PC wines produced by inoculation with yeast starter culture (Anchor NT202).

Alcohols are produced during alcoholic fermentation and play an important role in the flavor of wines, depending on the types of compounds and their concentrations *(Valero et al. 2002 & Selli et al., 2004)*. Higher alcohols are recognized by their strong and pungent smell and taste and they are related to herbaceous notes. At concentrations below 300 mg/l, they certainly contribute to the desirable complexity of the wine, but when their concentration exceeds 400 mg/l, higher alcohols are regarded as a negative factor on quality *(Selli et al., 2004 & Mateo et al. 2001)*.

4-vinyl Guaiacol is a volatile phenol which produced with biotransformation of the hydroxycinnamic esters (notably coumaric and ferulic acids) by Brettanomyces *(Chatonnet et al 1997)* and donates smoky, animal, stable-like notes. Off-odors are frequently detected when contents exceed 725 mg/liter. 4-vinyl Guaiacol was indentified at PC wine on low concentration.

A total of 6 fatty acids were identified in all the four wines. From the point of subtotal percentage, fatty acids were the least at PC wine and the highest at WC. Acetic acid is presented at W, WC and P wines with significant difference between them. On the other hand, PC is not included acetic acid. The amount of acetic acid is the main reason of subtotal percentage presenting every wine. It is clear that spontaneous alcoholic fermentation and spontaneous malolactic fermentation give more concentration of acetic acid than non-spontaneous fermentations. Decanoic acid was identified at W, P and PC. Fatty acids have been described with fruity, cheesy, fatty, and rancid notes *(Rocha et al. 2004)*.

The aroma compound 3-(methylthio)-1-propanol (methionol) has a powerful odour reminiscent of soup, meat, onions and potatoes and is derived from the sulphur amino acid methionine. In wine the methionine derivatives are considered as off-flavours (M.M.W. Etschmann et al. 2008). As can be observed in Table 5, W wine includes the highest amount of methionol, WC has the least and there is no difference to the concentration presented at P and PC samples. The odor threshold of 3-(methylthio)-1-propanol (methionol) is 1000 μg/L *(Guth, 1997).*

At a compare of wines (cabernet franc) in which MLF is carried out in steel tanks and then aged in oak barrels, with wines in which MLF has been carried out in barrels, one of the indentified volatile compounds was the Diidro-2(3H)-furanone (g -butyrolactone). This study showed that concentration of g -butyrolactone increases after MLF carried out *(P.M. Izquierdo-Canas et al., 2015)*. As shown in Table 5, at this study PC wine had the least concentration of g-butyrolactone in contrast with WC which had the highest. Between W and P there is no so big difference.

5.3 Sensory analysis

At the following table presents the results of sensory analysis. It is seen that the wines are almost all similar. Panelists did not recognize significant differences to the color and flavor of the wines. The only attribute which seemed to be affected by the use of the different fermentation starters was the “Fruity aromas”. The most references about a fruity character were for PC wine. This wine was inoculated with the starter NT202 and co-inoculated with *O. oeni* at the beginning of alcoholic fermentation.

Table 6: Sensory analysis results. The values shown correspond to the sum of the calculated ranks. The samples perceived as more intense for a given attribute, are those with the lower sum of the ranks (minimum significant difference between the ranks 17-p <0.05).

Wines	**Color intensity**		**Orange Hue**		**Fruity aromas**		**Vegetable aromas**		**Body-Structure**		**Astringency**		**General impression**	
W	33	a	32	a	37	b	27	a	30	a	34	A	27	a
WC	28	a	27	a	27	ab	27	a	28	a	27	A	30	a
P	24	a	35	a	35	ab	35	a	37	a	29	A	35	a
PC	35	a	26	a	26	a	31	a	25	a	30	A	28	a

5.4 Correlation between the results

Terpenes are widely distributed in grapes, and their content in grapes varies with different cultivars of V. vinifera. They can be thus described as primary aromas, or even varietal aromas. Terpenes exist in three forms: free monoterpene alcohols or oxides, glycosides (bound with sugars) and di- or triols. Although a total of 44 different terpenes have been identified in grapes, only 6 compounds in free form have aroma significance in wines *(Ribereau-Gayon et al., 2000)*. As shown in Table 8, there is not any correlation between identified free terpenes and fruity aroma. This means that fruity aroma of PC wine is not due to the free terpenes.

Table 8 : Correlations between of identified free terpenes and fruity aromas. Significant correlations ($p < 0.05000$) are in red color.

Variable	**Fruity aroma**	
		Limonene
Fruity aroma	1,000000	-0,787398
Limonene	-0,787398	1,000000
		Linalool
Fruity aroma	1,000000	-0,731549
	-0,731549	1,000000
		a-bergamotene
Fruity aroma	1,000000	-0,163035
a-bergamotene	-0,163035	1,000000
		terpinene-4-ol
Fruity aroma	1,000000	-0,509567
terpinene-4-ol	-0,509567	1,000000
		β-citronellol
Fruity aroma	1,000000	-0,010068
β-citronellol	-0,010068	1,000000
		Geraniol
Fruity aroma	1,000000	0,503744
Geraniol	0,503744	1,000000
		caryophyllene oxide
Fruity aroma	1,000000	-0,509567
caryophyllene oxide	-0,509567	1,000000
		Farnesol
Fruity aroma	1,000000	-0,405943
Farnesol	-0,405943	1,000000

		3-oxo-a-ionol
Fruity aroma	1,000000	-0,871717
3-oxo-a-ionol	-0,871717	1,000000

As is well-known, most esters have the typical fruity and floral aroma of young wines *(Francioli et al., 2003)*. Fatty acids (C6 to C10) at concentrations of 4 to 10 mg/L impart mild and pleasant aroma to wine. However, at levels beyond 20 mg/L, their impact on wine becomes negative *(Shinohara et al.1985)*. Acetic acid was the major fatty acid found in three of four wines. Acetic acid is produced during alcoholic and malolactic fermentation. At low levels this compound lifts wine flavors, however, at high levels; it is detrimental to the taste of wine by leaving the wine tasting sour and thin (Joyeux A. et al., 1984*)*. As shown in Table 9, there is not any correlation between identified aroma compounds and fruity aroma.

Table 9 : Correlations between of identified aroma compounds and fruity aromas. Significant correlations ($p < 0.05000$) are in red color.

Variable	**Fruity aroma**	
		Diethyl succinate
Fruity aroma	1,000000	0,308776
Diethyl succinate	0,308776	1,000000
		Ethyl hexanoate
Fruity aroma	1,000000	0,051917
Ethyl hexanoate	0,051917	1,000000
		Ethyl hexadecanoate
Fruity aroma	1,000000	-0,527886
Ethyl hexadecanoate	-0,527886	1,000000
		Ethyl 3-Hydroxybutanoate
Fruity aroma	1,000000	0,767939
Ethyl 3-Hydroxybutanoate	0,767939	1,000000
		Ethyl 4-hydroxybutanoate
Fruity aroma	1,000000	-0,791220
Ethyl 4-hydroxybutanoate	-0,791220	1,000000
		Ethyl lactate
Fruity aroma	1,000000	0,710068
Ethyl lactate	0,710068	1,000000

		Ethyl octanoate
Fruity aroma	1,000000	0,111723
Ethyl octanoate	0,111723	1,000000
		2-Phenethyl acetate
Fruity aroma	1,000000	-0,666136
2-Phenethyl acetate	--0,666136	1,000000
		2- and 3-methyl-1-butanol
Fruity aroma	1,000000	0,004727
2- and 3-methyl-1-butanol	0,004727	1,000000
		1,3-Butanediol
Fruity aroma	1,000000	-0,024108
1,3-Butanediol	-0,024108	1,000000
		2,3-Butanediol
Fruity aroma	1,000000	0,354163
2,3-Butanediol	0,354163	1,000000
		cis-3-hexen-1-ol
Fruity aroma	1,000000	-0,155752
cis-3-hexen-1-ol	-0,155752	1,000000
		2-Ethyl-1-hexanol
Fruity aroma	1,000000	-0,629465
2-Ethyl-1-hexanol	-0,629465	1,000000
		1-Hexanol
Fruity aroma	1,000000	0,540887
1-Hexanol	0,540887	1,000000
		2-Methyl-1-propanol
Fruity aroma	1,000000	0,587610
2-Methyl-1-propanol	0,587610	1,000000
		2-Phenylethanol
Fruity aroma	1,000000	-0,538659
2-Phenylethanol	-0,538659	1,000000
		1,2-Propanediol
Fruity aroma	1,000000	-0,629465
1,2-Propanediol	-0,629465	1,000000
		4-Vinylguaiacol
Fruity aroma	1,000000	-0,629465
4-Vinylguaiacol	-0,629465	1,000000
		Acetic acid
Fruity aroma	1,000000	0,408956
Acetic acid	0,408956	1,000000
		Decanoic acid
Fruity aroma	1,000000	0,351812
Decanoic acid	0,351812	1,000000
		Hexanoic acid
Fruity aroma	1,000000	-0,426861
Hexanoic acid	-0,426861	1,000000

		3-Methylbutanoic acid
Fruity aroma	1,000000	-0,519089
3-Methylbutanoic acid	-0,519089	1,000000
		2-Methylpropanoic acid
Fruity aroma	1,000000	-0,503653
2-Methylpropanoic acid	-0,503653	1,000000
		Octanoic acid
Fruity aroma	1,000000	0,846288
Octanoic acid	0,846288	1,000000
		Diidro-2(3*H*)-furanone (g - butyrolactone)
Fruity aroma	1,000000	0,216687
Diidro-2(3*H*)-furanone (g - butyrolactone	0,216687	1,000000
		3-(methylthio)-1-ropanol (methionol)
Fruity aroma	1,000000	0,743619
3-(methylthio)-1-ropanol (methionol)	0,743619	1,000000

5.5 Conclusion

The obtained results indicated that the choice of yeast and bacteria affects the volatile composition and sensory properties of wine. The outcomes clearly indicate some differences between spontaneous and non-spontaneous fermentation. The spontaneous alcoholic fermentation produces higher volatile content and more terpenes. The combination of spontaneous alcoholic fermentation and non-spontaneous MLF (WC) in contrast with spontaneous alcoholic and MLF fermentation (W), increases the concentration of many free terpenes, gives two more terpenes (caryophyllene oxide, terpinene-4-ol), decreases concentration of ethyl esters, higher alcohols and 3-(methylthio)-1-ropanol (methionol), doesn’t produces ethyl 3-hydroxybutanoate but ethyl 4-hydroxybutanoate, gives the highest concentration of acetic acid, does not produces decanoic acid and show the highest amount of diidro-2(3H)-furanone (g -butyrolactone). In the cases of non-spontaneous alcoholic fermentation there are differences between spontaneous (P) and non-spontaneous MLF (PC) fermentation. PC wine has more terpenes (limonene, linalool, farnesol and 3-oxo-a-ionol), higher concentration of ethyl esters, more amount of higher alcohols including 2-ethyl-1-hexanol, 1,2-propanediol and 4-vinylguaiacol, less concentration of diidro-2(3H)-furanone (g -butyrolactone) and does not produces acetic acid. P is the wine with the least volatile content. PC gave the best results concerning of intensity of fruity aroma. Except of fruity aroma, panelists did not recognize any other significant differences between wines, confirming that also spontaneous fermentation gave good results in the experimental conditions tested. This is an interesting fact, considering that all fermentation trials were carried out without sulfur dioxide supplementation, and only 50 mg/L were added to the wines at bottling. Between the indentified terpenes there are five sesquiterpenes, a-bergamotene, caryophyllene, caryophyllene oxide, farnesol and a-copaene. Caryophyllene oxide was found in the free fraction of WC wine and in bound fraction of W and WC. a-Copaene was found in bound fraction of W. This is the first time that these compounds identify in wine. Further experiments, shall keep into account how wines will undergo chemical and sensory modifications during storage and ageing.

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Wide side

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