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# "Technological Innovations applied to the Winemaking Tradition"

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# *PREFACE*

In the world of wine, tradition is a rooted component evoking authenticity of the product and fascinating the consumer. Today's winemakers combine knowledge of the past with modern technologies to obtain a wine without defects and with peculiar attributes. Technological advances, however, require the study of their impact on a complex product such as wine with the aim of guaranteeing progress in the quality of the final product.

The use of modern technologies applied to material and methodologies typical of the winemaking tradition are investigated in this work. The analyzes performed would like to provide helpful insights for extending the knowledge about the changing occurring in wine.

Especially, this PhD thesis investigates three different aspects of winemaking:

I) The impact of the type of container used during and after the fermentation: the study focused on the comparison between wines obtained with earthenware amphorae and wooden containers.

II) The influence of the type of stopper during the storage in bottle: the investigation concerned the comparison between wines closed with a new type of stopper made of natural material without glue addition and wines closed with conventional cork-based stoppers.

III) The binding of cyclic and non-cyclic PACs to potassium and calcium ions, which affect the chemico-physical stability of wines: novel cyclic proanthocyanidins recently discovered in wine, are investigated in the chemical properties to evaluate their impact on the colloidal stability of wine.

In this regard, the thesis is organized in three chapters related to the three issues abovementioned.

In each chapter an overview of the topic based on bibliographic research is followed by the description of the experimental plan and the methodologies adopted. Results are reported along with discussion followed by the final conclusion.

The general conclusion of this PhD thesis is reported after the three chapters to summarize the results obtained and to suggest future perspectives.

# ABSTRACT

**CHAPTER I**

The investigation aimed at evaluating the effects of in-amphorae winemaking process with comparison to the classic white wine process. Phenolic, volatile profiles and sensory analysis of Chardonnay wines obtained with three different storage systems (large wooden tank, small toasted barrique and earthenware amphorae) were determined. The chemical and sensory results of the three wines were statistically elaborated in order to point out the possible influence of the containers during the winemaking and the storage period prior to bottling.

The results showed that in-amphorae wines had more abundant catechin and caffeic acid and less abundant caftaric acid and trans-coutaric acid. Among the volatile compounds, higher alcohols contributed more for the in-amphorae wine. The sensory analysis revealed that four variables could distinguish wines made in-amphorae compared with the other containers: solvent and acetone, astringent/pungency, fruity, and color intensity. The results provided knowledge for the possible development of a new Chardonnay wine-style obtained through earthenware amphorae.

**CHAPTER II**

The aims of this work were (1) The assessment of the impact of different types of stoppers on the chemical and sensory parameters of four wines from South Tyrol – Alto Adige region (2) To provide knowledge on the evolution of phenolic and volatile compounds during a bottle storage period of twelve months. In these analyzes appeared that the non-anthocyanin phenolic compounds named gallic acid, caffeic acid, p-coumaric acid, trans-resveratrol, GRP and protocatechuic acid had a common evolution trend in the four wines. The volatile profile was characterized by the high concentrations of isopentyl acetate, 1-hexanol, ethyl hexanoate, 2-phenylethyl alcohol, diethyl succinate, ethyl octanoate and ethyl decanoate. During the bottle storage period the modification of the volatile composition was primary due to the evolution of these compounds.

Two-way ANOVA performed on the phenolic and volatile compounds showed a large variability of the statistical significance between the two types of stoppers. It was not highlighted a common trend that could relate the type of stopper with the phenolic and volatile compounds. Besides, the statistical results highlight the dominant influence of the storage time over the type of stopper on the phenolic composition. Multivariate statistic pointed out the similarities between the two types of stoppers. The comparison between different types of stoppers allowed a better comprehension of their influence on the final product.

**CHAPTER III**

This last chapter studied the binding of cyclic and non-cyclic proanthocyanidins to potassium and calcium ions, which affect the chemico-physical stability of wines. Nineteen red and white wines were analyzed by HPLC/HRMS/MS with positive electrospray ionization to investigate the distribution of novel cyclic PACs and their calcium, potassium and sodium adducts. Principal components analysis was used to study the distribution of the wines and the relationships among PACs with and without cation complexes. A dependence on specific isomers (and conformations) was found for the non-cyclic procyanidin (PC) trimer whereas the cyclic tetrameric PACs were shown to bind better to potassium than their non-cyclic analogues. The binding to these metals appeared to be influenced not only by the number of monomer units, but also by the conformation assumed by the molecules. The multivariate analysis of the mass-spectrometric results showed a relationship with the grape variety which allowed the proposal that their relative abundances could be used as tools for differentiating the wines by grape variety.

## Contents

# CHAPTER 1

# Effect of the winemaking in Amphorae on the chemical and sensory profile of a Chardonnay wine

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## 1.1 Introduction

Earthenware is a very porous material which has been used for the storage of wine and olive oils since ancient times. Firstly, it was adopted in the Middle East (Pecci *et al.*, 2013) whereas In Italy, it was used since ancient Rome, and there is evidence of the production of amphorae destined to wine dated to the IV-V century A.D.

However, making wine in a permeable container such as amphorae led to excessive oxidation of the final products. Often, wine was added with honey and spices in order to mask the acetic off-flavour, the off-taste and could not be stored in amphorae for long periods. So, with the end of antiquity, the use of ceramic containers disappeared definitively from the Italian peninsula. Eventually, wooden containers replaced the earthenware for good, and they have been the main vinification tanks adopted until the modern age. Wood is generally less porous than clay, less heavy and thus wooden containers were preferred to clay for the storage and the transportation of goods.

Ceramic (from the ancient Greek "Kéramos" which means clay) defines an inorganic, nonmetallic material, malleable in natural state, stiff after firing. Usually it is composed by different materials: clays, feldspars (sodium feldspars, potassium feldspars or both), silica sand, iron oxides, alumina, quartz. Such an articulated mix determines the presence of flattened molecular structures, called phyllosilicates. Their shape, in the presence of water, gives the clay some plasticity and makes processing easier and more successful (amphoraeforwine.com n.d.).

The permeability of clay is a factor that can be determined by two factors: the composition of the material used for its construction and the cooking temperature to which the amphorae is subjected. The natural porosity of the clay allows to oxygenate the content of the amphora in a similar way to the characteristic exchange processes of the wood without making any organoleptic cession. This ability to oxygenate can be varied according to the needs of the wine maker by acting on the cooking temperatures of the amphora. The latter parameter results to be inversely proportional to the absorption of water. If an amphorae is cooked at a temperature of 1000 °C, it will have a permeability of 12-14%, while an 1100 °C will be 2-0%. In the interval between these two temperatures, of 100 °C, it is possible to decide how much the amphorae is able to absorb. This allows to create specific containers for each use,

adapting the amphorae to the different types of grapes and to the different winemaking practices depending on the product that must contain.

Nowadays, the processing technology of earthenware has much improved, and it allows slower oxygenation rates than in the past. The main aim of in-amphorae winemaking is to provide a beneficial oxygen microdiffusion without the transfer of wooden aroma compounds (such as vanillin, tannins, and toasted flavors) common to vinification in wood tanks (Baiano *et al*, 2014). Consequently, the recent resumption of using modern earthenware amphorae for winemaking comes from the desire of vintners to rediscover old processing and storage techniques, by adopting traditions disappeared for several centuries (Baiano *et al*, 2014; 2015). Nowadays, several wineries are combining a traditional in-amphorae winemaking methods in combination with modern technologies such as the addition of dry-ice or other cooling devices protecting the grapes and the must. The resulting wine had peculiar characteristics and has been proposed with the purpose of attracting new groups of consumers

Although the historical tradition of winemaking in-amphorae is well known, there is only little scientific information regarding the effects of in-amphorae winemaking on the quality, the chemical profile, and the sensory properties of wines obtained with this technique. According to Lanati *et al* (2010) Georgian white wines aged in amphorae had a darker, almost orange color, which is uncommon for white wines. Recently, Baiano *et al* (2014; 2015) performed some researches on white wines produced with modern amphorae. The results obtained for the Falanghina wine showed that the wines aged in glazed and engobe amphorae had a similar evolution of physico-chemical indices. Engobe amphorae allowed the best retention of phenolic compounds, especially flavans reactive with vanillin compared to raw and glazed amphorae. Other studies performed on Minutolo wine showed the dramatic reduction of flavonoids and flavans reactive with vanillin in the case of raw amphorae. The highest antioxidant activity was exhibited by wines in engobe amphorae, whereas the lowest values were showed by the wines in glazed amphorae.

The aim of this work was to compare the effects of the winemaking process on Chardonnay wine with three different storage systems (barrel, barrique, and amphorae). The chemical determinations and sensory results were statistically

elaborated in order to point out significant differences and/or similarities between the three different Chardonnay wines, which were analyzed from the fermentation until bottling after a six-month storage period. Moreover, the study investigated on the effects of in-amphorae winemaking on the phenolic and volatile profiles on the obtained Chardonnay wine after one year of bottle storage. The use of mass spectrometry (MS) coupled to ultrahigh-performance liquid chromatography (UHPLC) and gas chromatography (GC) was a powerful tool to detect, identify, and quantify the characterized phenolics and volatile compounds of three winemaking methods (earthenware, large wooden tanks, and small toasted barrels). The results are compared with the sensory preferences to provide a better analytical and technological knowledge potentially applicable to the winemaking strategies of a winery.1.

## 1.2 Material and Methods

The Chardonnay grapes were harvested on August 31, 2014 in a single vineyard of 7 ha located in S. Venanzio di Fossombrone (PU, Italy). The vineyard was planted in 2007 and the vine training system was Guyot. An amount of 80 q of grapes was harvested manually in a single day and was destined to three different types of vinification: 2 barrels (2000 l each), 3 barriques (225 l each), and 2 amphorae (225 l each). The quality profile of the wines was monitored in the first six months of the winemaking process from the chemical and sensory point of view. In the area of Fossombrone, the average minimum and maximum temperatures of the last 30 years in June and August are about 15–18°C and 23 –27°C, respectively. In 2014, the average minimum and maximum temperatures were 16.2–18°C and 24.9–26.6°C in the same months, thus they were in average with the climatic values. The average relative humidity was ca. 60% and there were about ten rainy days for each month.

Winemaking in amphorae

The earthenware amphorae (225 l) were obtained from Tava s.r.l., Mori, Italy (Figure. 1) and had a porosity lower than 6%, water absorption of about 3.5%, pore diameter equal to about 0.05 μm, corresponding to a flow of $O_2$ of 0.4 ml/l/month, according to the producer.

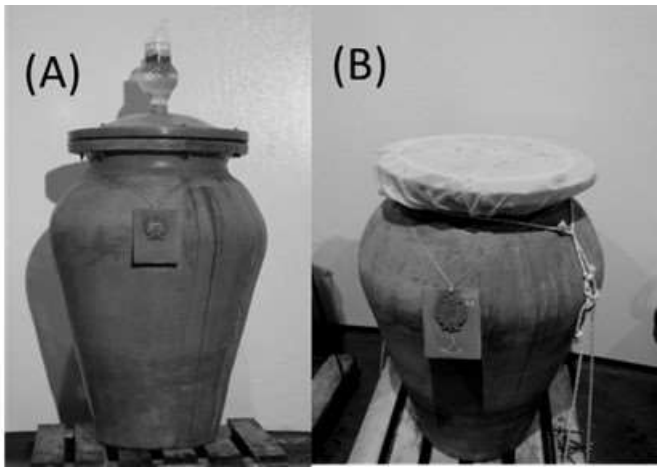



**Figure. 1**. Earthenware amphorae **(A)**, a cap of brushed cotton applied at the top of the amphorae **(B)**

An aliquot of Chardonnay grapes (about 8 q) was manually selected and sent to the destemmer where the air was replaced by nitrogen gas. The berries were separated from the stems and remained practically undamaged. Then, the berries were put manually into the two amphorae. A yeast culture of *Saccharomyces cerevisiae* (premium Chardonnay; VASON, Verona, Italy) was inoculated at 20 g/hl. Because of the strong development of carbon dioxide during the fermentation in amphorae and to avoid the contamination by insects or other sources, a 'cap' of brushed cotton was applied at the top of the container. Anyway, the cap was permeable to the fermentation gas. The progress of the fermentation was daily monitored using a Baumè hydrometer (Exacta+Optech, San Prospero, Italy). After about a week, the alcoholic fermentation ended and the malolactic fermentation was induced by inoculating lactic acid bacteria strains (*Oenococcus oeni*) at 1 g/hl (Viniflora; VASON). When the malolactic fermentation was over and the gas production stopped, dry ice was deposited on the top layer of pomace inside the amphorae in order to prevent oxidation. The amphorae were then closed and sealed with their cover through a silicone gasket for food use. The dry ice sublimated through the bunghole. Successively, carbon dioxide was flown through the bunghole 4–5 days after closing the amphorae with a flexible tube in order to assure an inert head space. The in-amphorae maceration lasted until March of 2015. Then, the whole mass of pomace was extracted from the top of the container with the aid of pipes and a pump and poured on a grill. The wine was maintained in a reduced atmosphere and was then transferred into a steel tank until bottling, which was carried out in May. Bottling was

manual. However, nitrogen gas was used to displace oxygen inside the bottle and in the headspace between the cork and the wine.

### *1.2.1 Winemaking in barrels and barriques*

The remaining 70 q of grapes were used for the other two vinifications, in barrels and in barriques, respectively. The berries were separated from the stems and crushed, still replacing the air with nitrogen, and were cooled up to 10°C through a concentric tubes heat exchanger of about 60 m length. The cooled berries were softly pressed by using a pneumatic press Velvet 50 (Diemme S.p.A., Lugo, Italy), with steps at increasing pressure values, each of about 0.2 bar, until a value of wine-to-grape yield of about 72% was reached. Then, the juice was moved into an underlying tank and was continuously maintained in inert atmosphere conditions with nitrogen gas. Afterwards, the juice was transferred into a steel tank of 80 hl capacity equipped with a cooling system. The juice was decanted for about 34 h at a temperature of 12°C. After decantation, the juice was further clarified through flotation and subsequent removal of the liquid from the lower valve of the tank with a pump. The clarified must was transferred to another steel tank of the same capacity. Afterwards, the tank was heated to 18°C and inoculated with *Saccharomyces cerevisiae* (20 g/hl) (Premium Chardonnay; VASON) and inactivated yeast (30 g/hl) (B-vitality; HTS, Marsala, Italy) was added as nutrients. The must was then divided into two large non-toasted oak barrels, each 20 hl, and in 3 toasted oak barriques of 225 l. At the end of the alcoholic fermentation, the malolactic fermentation was induced with the inoculation of lactic acid bacteria of the type *Oenococcus oeni* (1 g/hl) (Viniflora; VASON) before closing the bunghole. For the first three months, bâtonnage was carried out once a week; then, the frequency of mixing was halved in the next two months. Sulfur dioxide was added to the wine contained in barrels and barriques (25 mg/l) where the wine continued its aging in wood until May. Then the wine was transferred from the oak barriques and barrels into steel tanks, at a temperature of 3°C for ten days. After this period, the wine was immediately transferred in adjacent tanks at low temperature. The filtration was carried out through a filter press before bottling. The bottles were previously rinsed with sterile water and then dried with compressed nitrogen gas at 2 atm. The air was eliminated through a vacuum pump and the insufflation of

nitrogen (99.8%), by filling with a slight depression and automatic leveling. The head space of the bottle was saturated with nitrogen gas prior to insertion of the cork stoppers.

### *1.2.2 Analytical and sensory determinations*

The analytical determinations (Brix, titratable acidity, malic acid, and pH) were performed both in the Chardonnay grapes during the maturation (data not reported) and at the day of the harvest. The chemical determinations carried out in the wine were alcohol content (% vol.), total sulfur dioxide (mg/l), titratable acidity (g/l), volatile acidity (g/l), pH, malic acid (g/l), lactic acid (g/l), and dry extract (g/l). The wine was sampled during the winemaking process at four different time intervals before bottling: 17/9/2014, 28/11/2014, 17/2/2015, 13/3/2015. Bottling took place in May. The chemical analyses (except total sulfur dioxide) were conducted with a WineScan™ (FOSS, Padova, Italy) interferometer, which is based on the Fourier transform infrared spectroscopy (FTIR). The sulfites were determined by using an automatic $SO_2$ titrator ($SO_2$-Matic 23; Crison Instruments, S.A., Barcelona, Spain), based on the Ripper method (an automatized iodine titration). Iodide 0.01M, sulfuric acid ($H_2SO_4$) 25%, sodium hydroxide (NaOH) 4M, and solid potassium iodide (KI) were of analytical grade.

The sensory characteristics of the wines were evaluated through a panel formed by twelve trained judges (professors, researchers, and students). The wine was served at 12°C in ISO type tasting glasses (height 155 mm, glass diameter 65 mm, capacity, 215 ml) from Bormioli (Parma, Italy). The glasses were filled with 50 ml wine. The sensory descriptors evaluated by the judges were identified during the first session with the procedure of the round table: 'straw color', 'vanilla flavour', and astringent (tannin) perception. Each sample was evaluated by using a scale of four points (1 = no perception, 4 = highest intensity). The panel also formulated a final judgement of the three different finished wines.

**Table 1.** Chemical and sensory data of the three different wines monitored during the winemaking process and storage (amphorae, n=2; barrels, n=2; barriques, n=3). $SO_2$ tot, total sulfur dioxide; TA, total acidity; VA, volatile acidity; sd, standard deviation.

| | alcohol (% vol) | $SO_2$ tot (mg/l) | TA (g/l) | VA (g/l) | pH | Malic acid (g/l) | Lactic acid (g/l) | Dry extract (g/l) | vanilla | tannic | straw color |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 17.09.2014 | | | | | | | | | | | |
| amphorae | 12.55 | 9 | 6.4 | 0.23 | 3.27 | 1.27 | 0.66 | 26.2 | 1 | 1 | 2 |
| barrique | 12.7 | 18 | 6.5 | 0.22 | 3.28 | 1.42 | 0.48 | 22.4 | 1 | 1 | 2 |
| barrel | 12.65 | 18 | 6.6 | 0.23 | 3.28 | 1.71 | 0.31 | 21.37 | 1 | 1 | 1 |
| Average ± sd | 12.6± 0.08 | 15±5.2 | 6.5± 0.1 | 0.23±0.0 | 3.27±0.0 | 1.48± 0.22 | 0.48±0.17 | 23.3± 2.5 | 1±0 | 1±0 | 1.7± 0.6 |
| 28.11.2014 | | | | | | | | | | | |
| amphorae | 12.57 | 7 | 5.35 | 0.4 | 3.28 | 0.02 | 1.35 | 23.8 | 1 | 4 | 2 |
| barrique | 12.72 | 36 | 5.9 | 0.25 | 3.27 | 1.1 | 0.77 | 21.26 | 2 | 1 | 2 |
| barrel | 12.7 | 34 | 5.85 | 0.23 | 3.28 | 0.95 | 0.82 | 21.33 | 1 | 1 | 2 |
| Average ± sd | 12.7±0.1 | 25.7±16 | 5.7±0.3 | 0.29±0.1 | 3.28±0.01 | 0.60±0.6 | 0.98±0.32 | 22.1±1.4 | 1.3±0.6 | 2±1.7 | 2±0 |
| 17.02.2015 | | | | | | | | | | | |
| amphorae | 12.52 | 9 | 5.2 | 0.37 | 3.28 | 0.16 | 1.53 | 23.1 | 1 | 2 | 3 |
| barrique | 12.7 | 40 | 5.3 | 0.2 | 3.27 | 0.25 | 1.45 | 21.63 | 3 | 2 | 2 |
| barrel | 12.7 | 40 | 5.45 | 0.19 | 3.28 | 0.12 | 1.58 | 21.07 | 2 | 1 | 2 |
| Average ± sd | 12.6±0.1 | 30±18 | 5.3±0.1 | 0.25±0.1 | 3.28±0.01 | 0.18±0.1 | 1.47±0.1 | 21.75±1.0 | 1±1 | 1.7±0.6 | 2.3±0.6 |
| 13.03.2015 | | | | | | | | | | | |
| amphorae | 12.52 | 35 | 5.1 | 0.38 | 3.28 | 0.17 | 1.47 | 21.75 | 1 | 2 | 3 |
| barrique | 12.72 | 64 | 5.35 | 0.28 | 3.28 | 0.13 | 1.52 | 20.21 | 4 | 2 | 2 |
| barrel | 12.7 | 65 | 5.35 | 0.24 | 3.28 | 0.02 | 1.49 | 20.35 | 2 | 2 | 2 |
| Average ± sd | 12.6±0.1 | 55±17 | 5.23±0.1 | 0.30±0.1 | 3.28±0 | 0.11±0.08 | 1.49±0.02 | 20.77±0.8 | 2.33±1.5 | 2±0 | 2.3±0.6 |

### *1.2.3 Chemicals*

Water, methanol, and formic acid (all Optima LC/MS grade) for the UHPLC-MS analysis were obtained from Fisher Scientific (Geel, Belgium). Standard compounds (gallic acid, caffeic acid, (+)-catechin, and p-coumaric acid) used to confirm the identification of phenolics in wine were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France.

### *1.2.4 GC-MS determination of volatile compounds*

The GC-MS determination of volatile compounds was performed according to a published procedure (Rodrigues *et al.,* 2008) with slight modifications reported as follows. The wine was introduced into a 10-ml vial and closed with a screw cap equipped with an elastomeric septum. The vial was placed in a heating bath at 40°C for 10 minutes. Then a solid-phase microextraction fiber (divinylbenzene/carboxen/polydimethylsiloxane, 1 cm, 50/30 µm) from Supelco/Sigma-Aldrich (Milan, Italy) was introduced into the vial and exposed to the sample headspace for 15 minutes. The thermal desorption took place in the GC injector at 220°C for 15 minutes. The splitless injection (splitless time, 0.3 min) was performed in a Varian 3900 gas chromatograph coupled to a Saturn 2100T (Varian, Walnut Creek, California) ion trap mass spectrometer. The chromatographic separation was obtained with a ZB-5 capillary column (Phenomenex; 30 m × 0.25 mm I.D.; film thickness, 0.25 µm). The temperature program of the GC oven started at 40°C during 10 minutes and then was raised to 180°C at 3°C/min and reached 250°C at 15°C/min. The MS transfer line and trap temperatures were set at 200°C. The ion trap emission current was 10 µA. The mass spectra were recorded in the full scan mode (mass range, 31–250 m/z) at 1 scan per second.

Data were analyzed with the Varian Workstation software. The identification of volatile compounds was confirmed (1) with the GC retention index, (2) comparison with the NIST library mass spectra (Version 2.0; 2002), (3) injection of pure standard substances when available, and (4) with the aid of earlier reports (Cejudo-Bastante *et al.,* 2013; Ivanova *et al.,*2013 ; Hopfer *et al.,* 2012) Samples were analyzed in duplicate

(two different containers); peak areas were normalized over the total ion current of each sample and were reported as percentages.

### *1.2.5 UHPLC analysis of Phenolics*

The phenolic profile of the wines was obtained through UHPLC systems, an Agilent 1290 Infinity (at the University of Bordeaux) and a Shimadzu Nexera X2 (at the Free University of Bolzano-Bozen). Agilent system was equipped with a UV-vis diode array detector (DAD) (1290 Infinity) and connected to a quadrupole time of flight mass spectrometer (QToF/MS) with an electrospray ionization (ESI) source (Agilent 6530 Accurate Mass) run in negative ionization. The Shimadzu UHPLC was equipped with a fluorescence detector (Prominence RF-20A) and with a UV-vis photodiode array detector (SPD-M20A). The chromatographic separation was performed with a C18 UHPLC column (2.1 × 100 mm, 1.8 µm, Agilent). Water (Eluent A) and methanol (Eluent B) were used as mobile phases both acidified with 0.1% formic acid. The gradient program was as follows: 0% B for 0.5 minutes; 0% to 35% B for 19.5 minutes; 35% to 95% B for 4 minutes; 95% B for 3 minutes; 95% to 10% B for 1 minute; and 10% B for 2 minutes. The UHPLC flow rate was 0.3 ml/min, the injection volume was 2.0 µl, and the column temperature was set at 25°C. The mass spectrometer was operated in extended dynamic range of 2 GHz (m/z 3200). The nebulizer pressure and flow rate were set at 25 psi and 9 l/min, respectively. The drying gas temperature was 300°C. The sheath gas flow and temperature were set at 11 l/min and 350°C. The fragmentation, skimmer, OCT, and capillary voltage were at 150, 65, 750, and 4000 V, respectively. All the analyses were performed in negative mode. The chromatogram was recorded at the wavelength of 280 nm (quantitation wavelength). The identification of phenolics was achieved by comparison of their retention times and exact masses with those of the injected standard compounds. The quantitation was achieved using the diode array detector calibration curves of pure standard substances. When reference compounds were not available, a calibration with structurally related standard substances was used (gallic acid for protocatechuic acid; caffeic acid for caftaric acid, ethylcaffeate, and glutathionyl caftaric acid [GRP]; (+)-catechin for (–)-epicatechin; p-coumaric acid for cis-coutaric acid and trans-coutaric acid). The integration of the peaks allowed to obtain the concentrations of the

identified compounds. Concentrations are expressed in milligrams per liter of standard or of the structurally related standard.

### *1.2.6 Sensory evaluation*

The sensory characteristics of the wines stored for one year were evaluated by a panel formed by eight trained judges (professors, researchers, and students) at the University Department of Ancona. The wine was served at 12°C in ISO-type tasting glasses (height, 155 mm; glass diameter, 65 mm; capacity, 215 ml) from Bormioli (Parma, Italy). The glasses were filled with 50 ml of wine. The sensory descriptors evaluated by the panel were identified during the first session with the procedure of the round table: limpidity, color intensity, olfactory intensity, alcohol/liquor, vinegar, caramel/ toasted/cookie, herbaceous/green, fruity, tropical fruits, acid/citrus, alcoholic, sweet/honey, salty, wood/oak, herbaceous/unripe, solvent/ acetone, astringent/pungency, burning, and wine "body" perception were the sensory descriptors. Each sample was evaluated by using a scale of 10 points (1 = no perception, 10 = the highest intensity). The panel also formulated a final judgement for the three different wines.

### *1.2.7 Statistical analysis*

All the data obtained during six months of winemaking were analyzed by the univariate analysis of variance (ANOVA, $P < 0.05$) to determine which variables were statistically significant in order to differentiate the samples. In addition, also the correlation coefficients among the variables and the related P-values were calculated using GraphPad Instat (v.1.0 software, 2005, San Diego, California).

Principal component analysis (PCA) was carried out to point out differences or groupings between the wines obtained in amphorae, barrels, and barriques and analyzed during a 6-month storage period.

The chemical data (volatile and phenolic compounds) obtained after one year of bottle storage were also analyzed by univariate analysis of variance; when differences were detected, Tukey multiple comparison test and an $\alpha = 0.05$ criteria were applied using GraphPad Instat (v.1.0 software, 2005, San Diego, California). Data were expressed as single measurement performed on each different bottle for the three

typologies of containers (amphorae, A1 and A2; large wooden non toasted barrels, T1 and T2; and wooden toasted barrels, B1 and B2). Principal component analysis (PCA) was performed using The Unscrambler software (Camo Inc, Corvallis, Oregon). Hierarchical cluster analysis (HCA) was carried out using a single linkage algorithm and Euclidean distance with PAST software V 3.18 (Hammer & Harper, Oslo, Norway).

## 1.3 Results and Discussion

For the entire mass of Chardonnay grapes used in the experiment, the mean values of sugar content, acidity, malic acid, and pH were 21.9°Brix, 7.60 g/l, 2.25 g/l, and 3.17, respectively. From these results, the Chardonnay grapes were already mature and suitable for the harvest at the end of August because the potential alcohol content was 12.50% vol. and the titratable acidity was not too high for a correct winemaking process (it should be usually less than 10 g/l for still wines). This resulted in a moderate content of malic acid. However, very different wines were obtained from the same raw material, consisting of a batch of Chardonnay grapes, harvested in the same vineyard and in the same day but processed in different ways. Table 1 shows the chemical and sensory results of the three different wines monitored during the winemaking process and stored until the next May. The Chardonnay wine composition may vary to a large extent according to the maturity stage and hygienic state of the grapes, the geographical origin, and the winemaking practices. Cozzolino *et al.* (2003) and Stummer *et al.* (2005) reported a pH range of 3.0–3.4, a titratable acidity of 6.6–7.1 (g/l as tartaric acid), a volatile acidity of 0.20–0.40 (g/l as acetic acid), and a dry extract of 25.3 g/l for Chardonnay wines which were not processed in amphorae. These data are compatible with the results reported in Table 1, taking into account the different geographical origin and processing technology of the wines.

### *1.3.1 Analysis of variance of the chemical parameters during the winemaking*

The univariate ANOVA performed using all the data reported in Table 1 (including all the sampling times) showed that only the dry extract and volatile acidity could statistically differentiate the wine samples according to the container at $P < 0.05$. The dry extract (Figure. 2A) was significantly different between amphorae and barrel (P =

0.0258). The volatile acidity (Figure. 2B) was significantly different between amphorae and barrique or barrel (P = 0.0152). Although other chemical and sensory parameters between the three types of wines were numerically different, no other significant differences were registered. However, the univariate approach is not completely suitable to describe a multivariable model. In fact, the chemical or sensory variables which could well describe the variance of the samples at the beginning of the aging (September 2014) might not be able to differentiate the wines during or at the end of the sampling period (March 2015), or vice versa. Thus, a multivariate approach was studied, such as the model elaborated by using the PCA.

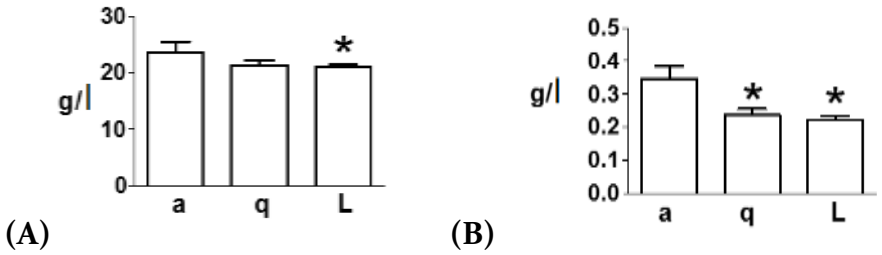



**Figure 2**. **(A)** Dry extract comparison between amphorae (a), barrique (q) and barrel (L); **(B)** volatile acidity comparison between amphorae (a), barrique (q), and barrel (L).

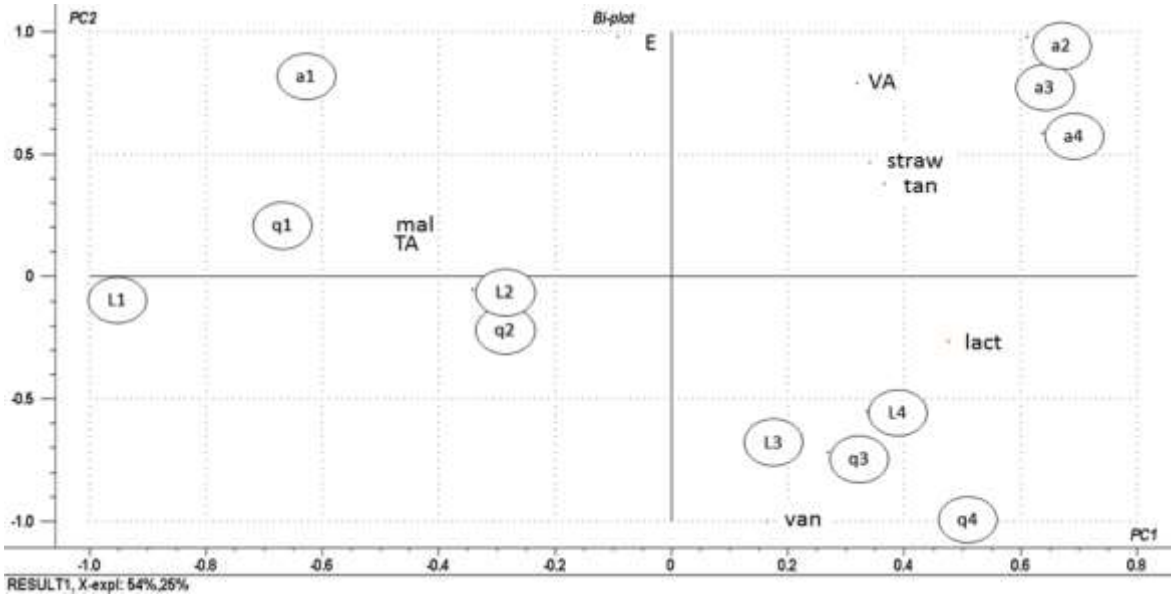
* significant difference between amphorae wine samples and selected samples *(p<0.05)*.

### *1.3.2 Principal component analysis*

The PCA was carried out in order to get a better overview of the effects of different winemaking procedures on the quality of semi-finished and finished Chardonnay wines in relationship with the aging period. The data (samples and variables) used for the multivariate analysis are a subpopulation of those reported in Table 1. In fact, the variables which remained unchanged or showed a negligible variation (alcohol, pH) were excluded from the model. Sulfur dioxide was also excluded because it is a variable more related to the winery practices rather than to the storage conditions. The bi-plots reporting PC1 vs PC2 and PC1 vs PC3 are displayed in Figure 3. The first two principal components (PC1 and PC2) accounted for 79% of the total variance of the model (Figure. 3A). PC1 was positively correlated with volatile acidity, tannin, lactic acid, and straw colour and negatively correlated with titratable acidity and malic acid. PC2 was positively correlated with the dry extract and negatively correlated with the vanilla flavour (Figure. 3B). The distribution of the samples was

strongly influenced by the storage time: all the samples 1 and 2 (except a2) were located in the left part of the plot, whereas the samples 3 and 4 were gradually located in the right quadrants. All the 'a' samples (amphorae) showed a peculiar distribution. The amphorae wines 2–4 were clearly differentiated from the analogue barrel and barrique wines due to the high volatile acidity, straw colour, and tannic perception. Barrel and barrique wines showed a higher vanilla flavour than the similar amphorae wines, presumably due to the storage in wood, as reported by Herrero *et al*. (2016). The dry extract of Chardonnay wines decreased from the first sampling carried out on September 17, 2014 to the last sampling on March 13, 2015, differently from Baiano *et al*. (2014), where the dry extract remained constant up to 6 months. However, the Chardonnay wine obtained in amphorae with maceration showed a remarkably higher final dry extract than the wine obtained from barrels and barriques. This was presumably due to the contact with the pomace, which led to the diffusion of extractable components including colouring substances, tannins, organic acids, salts, glycerol, and colloids. As expected, the malic acid content and titratable acidity decreased during the storage of all the wines.

**(A)**



**(B)**

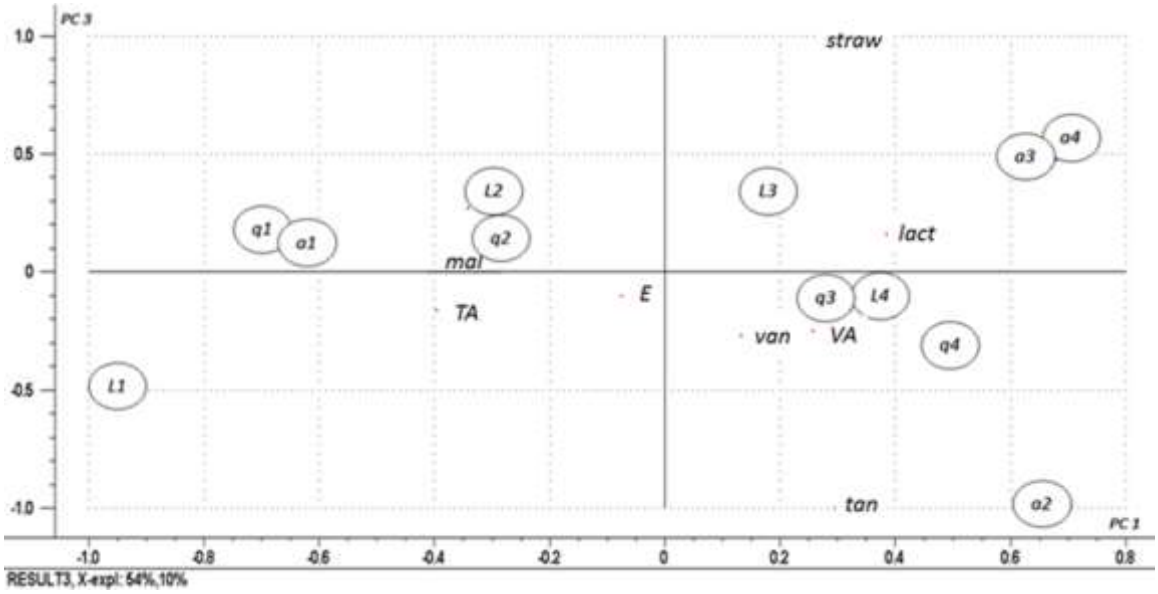



**Figure. 3**. Principal components analysis (PCA) of the Chardonnay wines: PC 1 vs PC 2 **(A)**, PC 1 vs PC 3 **(B)**

a = amphorae, q = barrique, L = barrel, tan = tannin, mal = malic acid, van = vanilla flavour, VA = volatile acidity, lact = lactic acid, E = dry extract, straw = 'straw' color, TA = titratable acidity; 1, 2, 3, 4 = time of sampling (respectively: 17/9/2014, 28/11/2014, 17/2/2015, 13/3/2015)

The amphorae wine showed lower titratable acidity (even if this difference was not significant) than the other two types of wines all through the storage. Presumably, a higher potassium extraction took place from the pomace during the maceration resulting in higher tartaric precipitation. Barrels and barriques showed almost the same values all through the storage period. Potassium metabisulphite was added immediately after the malolactic fermentation in barrels and barriques. Initially, the volatile acidity increased with a peak, then it was stabilized at values of around 0.4 g/l. The barrels maintained almost constant values, while barriques showed higher values than barrels. The volatile acidity was presumably influenced by the low sulfite content and permeability of the container, which enabled the wine contact with oxygen, resulting in higher production of acetic acid. The malolactic fermentation was closely dependent on the inoculation of *Oenococcus oeni*. In the present work carried out on Chardonnay, the main difference was the higher rate of conversion of malic acid into lactic acid observed in amphorae with respect to barrels and barriques, presumably due to the maceration with the pomace. Whereas, according to Baiano *et*

*al.* (2014) the organic acids like tartaric acid and malic acid showed no differences between the different types of Falanghina wine at the beginning and at the end of the storage obtained from amphorae and stainless still tank. In our study, the transformation of malic acid into lactic was much faster in amphorae than barrels and barriques. This trend could be due to the contact of the wine with the pomace during fermentation in amphorae, compared to barrels and barriques; the skins contain wild species of lactic acid bacteria, capable of completing the malolactic fermentation. The barriques needed more time for the conversion of malic acid into lactic acid, probably because of the bacteriostatic effect of the tannins present in the wood. However, the values of lactic acid detected in wines obtained from the three winemaking techniques did not vary very much at the end of the process.

### *1.3.3 Sensory analysis*

Apart from the average sensory results reported in Table 1 and in Figure 3, the sensory panel also expressed a final judgement on the three different Chardonnay wines. According to the panel, the wine produced in amphorae resulted to have a mature scent, a less 'green' character than wines from barrels and barriques, but a weak varietal aroma probably due to excessive maceration. The tannic content of amphorae Chardonnay was remarkable: it was made of elegant tannins, a pleasing taste which was higher than in the other wines. The panelists perceived a spicy scent, which was the index of a good maturation of the wine and did not resemble vanilla notes. The wine produced in barrique was characterized by an aromatic profile with 'vanilla notes'. However, the flavour profile easily evoked a wine obtained from Chardonnay grapes. This wine revealed characteristics of freshness, harmony, and a remarkable woody flavour. The panelists suggested to blend the barrique wine with other types of wine in order to reduce its high woody and vanilla notes. The barrel characterized the wine with fruity sensations corresponding to the Chardonnay grape variety. Among the three winemaking techniques, wine aged in barrel resulted in the most balanced product, with spicy and light woody and vanilla notes. It was characterized by a full, balanced, fruity, and persistent flavour. The panelists considered this wine as a good product to be potentially blended with the other two experimental Chardonnay wines. From the sensory point of view, the wine obtained in barrels

resulted to be the most 'complete' among the wines obtained from the three vinifications.

The analysis of correlations was performed using the data reported in Table 1 in order to point out the correlations among sensory and chemical variables. The alcohol content (not significantly different in the samples) and the total sulfites (which were directly added to the wine) were excluded from the elaboration. The color intensity of the wine was directly related to the volatile acidity (R = 0.607, P = 0.036) and the lactic acid content (R = 0.588, P = 0.044) and was inversely correlated with the titratable acidity (R = –0.653, P = 0.021). The tannic perception was linked to the extent of the malolactic fermentation, in fact it was directly related to the volatile acidity (R = 0.732, P = 0.0068), and inversely related to the malic acid content (R = –0.666, P = 0.018) and to the titratable acidity (R = –0.611, P = 0.035). The flavour of vanilla was not significantly correlated with the chemical variables, because the type of tank had a stronger influence than the chemical variables.

### *1.3.4 Volatile compounds*

The chemical profiles of the volatile compounds observed in the Chardonnay wine samples obtained in large non toasted oak barrels, in smaller toasted oak barrels, and in amphorae are presented in Table 2. Ethyl esters, such as ethyl hexanoate, diethyl succinate, ethyl octanoate, and ethyl decanoate were the most relatively abundant compounds in all samples. Other esters detected in all investigated samples were propyl butanoate; ethyl propionate; propanoic acid, 2-methyl-, ethyl ester; butanoic acid ethyl ester; butanoic acid, 2-methyl-ethylester; butanoic acid, 3-methyl-ethylester; 4-decenoic acid, ethyl ester; and ethyl dodecanoate. The alcohols present in all samples were 1-hexanol, 2-ethyl-1-hexanol, and phenylethyl alcohol. Other volatiles present in all samples were three furan compounds (compounds 9, 10, and 20 of Table 2). Other ubiquitous compounds were ionone and 1,2-dihydro-1,1,6-trimethyl-naphthalene. These results were in accordance with data reported by Cejudo-Bastante *et al.,* (2013) in which it was shown that diethyl succinate, phenylethyl alcohol, and ethyl hexanoate were the most dominant compounds in Chardonnay wine stored for 1 year. Furthermore, Ivanova *et al.,* (2013) reported that Chardonnay wines from Macedonia and Hungary possess the highest amount of total

esters in comparison with other red and white wines from the same regions. This is also in agreement with the relatively high esters content in this work (Table 2). Hopfer *et al.*, (2012) also reported a similar volatile profile in Chardonnay wines. The identification of the more volatile compounds with retention times (Rt) below 2.5 minutes was difficult due to the overlapping of several peaks. Therefore, these compounds were not suitable variables in the further statistical analysis.

**Table 2.** Volatile compounds identified by GC-MS in the wines produced in toasted wooden barrels (**B1** and **B2**), non-toasted wooden tanks (**T1** and **T2**) and clay amphorae (**A1** and **A2**) (relative percentages).

| No. | Compounds | Rt | Barrels | | Tanks | | Amphorae | | Base peak | Fragments |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *(min)* | **B1** % | **B2** % | **T1** % | **T2** % | **A1** % | **A2** % | *(m/z)* | *(m/z)* |
| **1** | n.i. | 1.67 | 5.23 | 4.47 | 4.29 | 7.83 | 10.91 | 7.91 | | |
| **2** | n.i. | 1.71 | 5.98 | 6.79 | 1.18 | | 1.61 | 1.94 | | |
| **3** | n.i. | 1.81 | 2.08 | 2.50 | | | 1.22 | 1.46 | | |
| **4** | n.i. | 1.86 | 1.98 | | | | | | | |
| **5** | n.i. | 2.38 | 4.69 | 4.35 | | 2.35 | 7.75 | 6.90 | | |
| **6** | Propyl butanoate | 2.55 | 1.13 | 1.07 | 2.99 | 2.00 | 1.81 | 5.38 | 89 | 71, 61, 43 |
| **7** | Acetic acid | 3.00 | | 0.16 | | | | | 60 | 43 |
| **8** | Ethyl propionate | 3.80 | 0.04 | 0.06 | 2.27 | 0.03 | 0.04 | 0.06 | 102 | 74, 57 |
| **9** | 2-Butyltetrahydrofuran | 4.66 | 29.22 | 18.87 | 13.23 | 14.67 | 31.79 | 20.74 | 71 | 55, 43 |
| **10** | Pantolactone | 5.06 | 0.01 | 2.86 | 3.18 | 3.17 | 2.78 | 9.55 | 71 | 57, 39 |
| **11** | Propanoic acid, 2-methyl-, ethyl ester | 5.15 | 0.24 | 0.22 | 0.18 | 0.22 | 0.11 | 0.14 | 116 | 88, 71 |
| **12** | 2-Pentanol | 6.28 | 0.06 | | | | | | 73 | 45 |
| **13** | Ethyl butanoate | 6.62 | 0.50 | 0.60 | 0.72 | 0.13 | 0.72 | 1.11 | 116 | 101, 88 |
| **14** | 2-Hydroxy-propanoic acid | 7.17 | 0.84 | 0.81 | | 0.73 | 1.06 | 91 | 73 | |
| **15** | Butanoic acid, 2-methyl-ethylester | 8.58 | 0.25 | 0.24 | 0.22 | 0.16 | 0.09 | 0.14 | 131 | 115, 102, 74, 57 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **16** | Butanoic acid, 3-methyl-ethylester | 8.72 | 0.40 | 0.42 | 0.45 | 0.30 | 0.18 | 0.27 | 131 | 115, 85, 57 |
| **17** | 1-hexanol | 9.49 | 0.60 | 0.53 | 0.45 | 0.54 | 0.78 | 0.89 | 84 | 69, 56, 43 |
| **18** | Isoamyl acetate | 9.72 | 0.72 | | 0.86 | 1.18 | 1.01 | 1.34 | 87 | 70, 55, 43 |
| **19** | Pentyl acetate | 10.16 | 0.07 | 0.76 | | | 0.24 | 0.51 | 87 | 70, 55, 43 |
| **20** | Furan, 2,2'-[oxybis(methylene)]bis- | 10.74 | 0.22 | 0.07 | 0.12 | 0.04 | 0.14 | 0.19 | 97 | 81, 69, 53 |
| **21** | 4-ethylbenzoic acid, 2-methylpropylester | 11.73 | 0.07 | | | | | | 163 | 151, 163 |
| **22** | n.i. | 12.32 | 4.34 | 3.2 | 3.86 | 3.46 | 3.75 | 5.26 | | |
| **23** | Ethyl hexanoate | 14.97 | 5.94 | 7.76 | 10.33 | 9.80 | 6.34 | 9.08 | 145 | 115, 99, 88, 43 |
| **24** | Limonene | 16.05 | | | | 0.05 | 0.09 | 0.29 | 136 | 121, 107 |
| **25** | 2-ethyl-1-hexanol | 16.19 | 0.38 | 0.59 | 0.32 | 0.83 | 1.01 | 0.97 | 83 | 70, 57, 41 |
| **26** | Ethyl-2-ethylhexanoate | 16.75 | 0.04 | | 0.07 | 0.05 | 0.01 | 0.01 | 99 | 73, 55 |
| **27** | Ethyl 2-furancarboxylate | 17.13 | | | 0.03 | 0.03 | | | 140 | 112, 95 |
| **28** | Pentyl isobutyrate (amyl isobutyrate) | 17.24 | | | 0.02 | | | 0.26 | 115 | 105, 70 |
| **29** | 2-nonanone | 18.65 | | | 0.03 | | | | 142 | 127 |
| **30** | Terpinolene | 18.51 | | | | | 0.05 | | 136 | 121, 105 |
| **31** | Linalool (t.i.) | 19.00 | | | | | 0.21 | 0.18 | 136 | 121, 105 |
| **32** | Phenylethanol | 19.48 | 1.62 | 2.28 | 0.94 | 0.96 | 2.44 | 2.01 | 121 | 103, 91 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **33** | Diethyl succinate | 22.07 | 8.48 | 9.67 | | 4.23 | 5.43 | | 12.32 | 10.29 | 101 | 129, 73, 55, 45 |
| **34** | Octanoic acid | 22.30 | 0.05 | 0.10 | | 0.03 | 0.29 | | | | 145 | 115, 87, 73 |
| **35** | Ethyl octanoate | 22.72 | 17.11 | 23.05 | | 32.58 | 28.14 | | 8.25 | 9.47 | 173 | 143, 129, 101 |
| **36** | Isopentyl hexanoate | 24.48 | 0.04 | | | 0.07 | 0.05 | | 0.03 | 0.03 | 143 | 117, 99 |
| **37** | Ionone | 25.52 | 1.07 | 1.21 | | 1.19 | 0.67 | | 0.59 | 0.68 | 192 | 177, 163, 149, 136, 121 |
| **38** | Ethyl nonanoate | 26.11 | 0.02 | 0.01 | | 0.02 | | | | | 157 | 143, 101, 88 |
| **39** | Naphthalene, 1,2-dihydro-1,1,6-trimethyl | 28.02 | 0.07 | 0.08 | | 0.08 | 0.03 | | 0.02 | 0.02 | 172 | 157, 142 |
| **40** | 4-Decenoic acid, ethyl ester | 29.13 | 0.12 | 0.13 | | 0.23 | 0.10 | | 0.09 | 0.11 | 199 | 169, 152, 135 |
| **41** | Ethyl decanoate | 29.43 | 5.83 | 6.61 | ***a*** | 14.52 | 15.04 | ***b*** | 2.32 | 2.5 ***c*** | 201 | 157, 171, 143 |
| **42** | Octanoic acid, 3-methylbutylester | 30.75 | | 0.06 | | | | | | | 171 | 145, 127 |
| **43** | Sesquiterpene (t.i.) | 31.09 | | 0.02 | | | | | | | 220 | 189, 177 |
| **44** | Ethyl dodecanoate | 32.21 | 0.76 | 0.45 | | 1.31 | 1.72 | | 0.24 | 0.2 | 229 | 199, 171, 157 |

Rt, retention time (min); n.i., not identified; % average concentration of the compounds (%); t.i., tentative identification.

Different letters indicate significant differences among the three containers used according to the Tukey test ($p < 0.05$).

### *1.3.5 Phenolic compounds*

The phenolic profile has been characterized by means of UHPLC-ESI(–)-QToF/MS. A typical chromatogram (in-amphorae wine sample, single wavelength monitoring at 280 nm) is shown in Figure 4. In all the three storage systems, eleven compounds were identified and were listed in Table 3. The peaks were numbered according to the elution order. The compounds identified were mainly hydroxycinnamic acids and their esters, namely, caffeic acid (CF), p-coumaric acid (PC), cis and trans-coutaric acid (CC and TC, respectively), caftaric acid (CT), glutathionyl caftaric acid (Grape reaction product - GRP), and ethylcaffeate (ET).

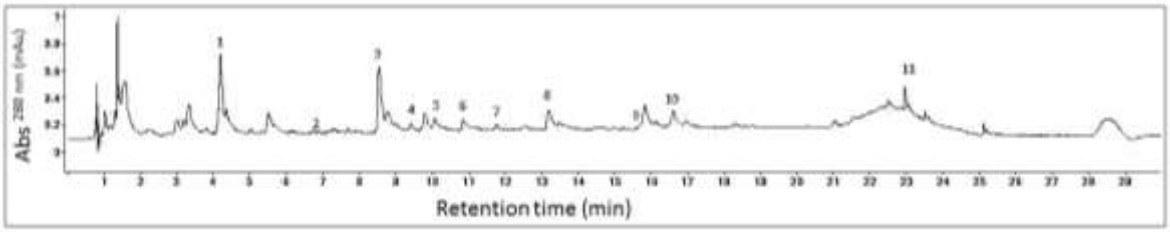



**Figure 4.** UV chromatogram (λ = 280 nm) of a Chardonnay wine with the identified peaks indicated and numbered. Peak assignments are reported in Table 3.

The identification of these compounds was achieved based on their retention times the experimental masses (m/z) of the deprotonated molecules. Gallic acid (GA), protocatechuic acid (PR), (+)-catechin (CA), and (–)-epicatechin (EC) were also detected, assigned, and quantified. In-amphorae wines (A1 and A2) showed more abundant (+)-catechin and caffeic acid and less abundant caftaric acid and trans-coutaric acid. Besides, there was a broad peak between 21 and 23 minutes, which could not be associated to any specific chemical component.

**Table 3.** Phenolic compounds identified by UHPLC-DAD-ESI(-)-QToF/MS in the wines produced in in toasted barrels (**B1, B2**), non-toasted wooden tanks (**T1, T2**) and clay amphorae **(A1, A2**).

| Peak no.[1] | Compound | Elemental composition (ion) | Rt *(min)* | ESI(-)-QToF/MS $[M-H]^-$ *(m/z)* | Exp. Acc. Mass $[M-H]^-$ *(m/z)* | Fragments *(m/z)* | Error *(mDa)* | Relative Concentration[2] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | **A1** | **A2** | **T1** | **T2** | **B1** | **B2** |
| 1 | gallic acid (GA) | $[C7H5O5]^-$ | 4.7 | 169.0195 | 169.0142 | 125.0266 | 5 | 12.7 | 11.8 | 18.3 | 14.6 | 4.5 | 7.6 |
| 2 | protocatechuic acid (PR) | $[C7H5O4]^-$ | 7.3 | 153.0210 | 153.0193 | 109.0308 | 2 | 0.3 | 0.6 | 0.0 | 0.0 | 0.3 | 0.0 |
| 3 | caftaric acid (CT) | $[C13H11O9]^-$ | 8.9 | 311.0458 | 311.0409 | 179.0380 - 149.0118 | 5 | 2.4 | 3.6 | 18.4 | 13.4 | 16.7 | 18.2 |
| 4 | glutathionyl caftaric acid (GRP) | $[C23H26N3O15S]^-$ | 9.7 | 616.1190 | 616.1090 | | 10 | 1.7 | 1.7 | 2.4 | 2.1 | 2.4 | 2.2 |
| 5 | *cis*-coutaric acid (CC) | $[C13H11O8]^-$ | 10.3 | 295.0495 | 295.0459 | 163.0424 - 119.0528 | 4 | 0.5 | 0.4 | 1.2 | 0.9 | 1.4 | 1.5 |
| 6 | *trans*-coutaric acid (TC) | $[C13H11O8]^-$ | 11.1 | 295.0494 | 295.0459 | 163.0428 - 119.0521 | 4 | 0.7 | 0.6 **a** | 1.6 | 1.7 **b** | 2.1 | 2.3 **c** |
| 7 | (+)-catechin (CA) | $[C15H13O6]^-$ | 11.9 | 289.0760 | 289.0718 | | 4 | 10.4 | 4.3 | 2.5 | 2.9 | 1.0 | 0.7 |
| 8 | caffeic acid (CF) | $[C9H7O4]^-$ | 13.4 | 179.0381 | 179.0350 | 135.0476 | 3 | 15.3 | 10.4 | 4.6 | 4.6 | 2.9 | 3.3 |
| 9 | (-)-epicatechin (EC) | $[C15H13O6]^-$ | 15.8 | 289.0793 | 289.0718 | 245.0896 | 8 | 2.1 | 2.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 10 | *p*-coumaric acid (PC) | $[C9H7O3]^-$ | 16.8 | 163.0419 | 163.0401 | 119.0524 | 2 | 4.9 | 4.5 | 0.9 | 1.6 | 0.6 | 0.6 |
| 11 | ethylcaffeate (ET) | $[C11H11O4]^-$ | 22.9 | 207.0690 | 207.0663 | 179.0373 - 161.0268 | 3 | 0.0 | 0.0 | 1.9 | 3.3 | 1.9 | 1.8 |

[1] referring to the chromatographic trace of Figure. 4.

2 eq. mg/l of the relative standard.

1 Rt. retention time (min)

Different letters indicate significant differences among the three containers used according to the Tukey test ($p < 0.05$).

### *1.3.6 Statistical analysis of the chemical data after one year of bottle storage*

Previous reports showed that volatile compounds are suitable variables to differentiate white wines stored under different conditions in raw, glazed, and engobe amphorae (Baiano *et al.,* 2014; 2015). They were also influenced by the different levels of toasting in wooden vinifications (Herrero *et al.,* 2015). Herein, statistical analysis was applied to the chemical data to identify markers for the different vinifications used. For exploratory data analysis, data pretreatment is a useful practice to avoid trivial conclusions (Berrueta *et al.,* 2007). Analysis of variance was used to assess if some differences in the aroma and phenolic compositions were statistically significant to discriminate the samples (six samples: A1, A2, B1, B2, T1, and T2) according to the three different storage materials used. The chemical variables evaluated with analysis of variance were the phenolic compounds listed in Table 3 (11 compounds) and the aroma compounds reported in Table 2 (44 compounds), with the exception of five unidentified compounds eluted in the first 2.5 minutes of the chromatogram. These compounds were excluded from the analysis due to their too high volatility and since they overlapped in a short elution interval in the early chromatogram. Analysis of variance showed that only one volatile (ethyl decanoate, 41) and one phenol (trans-coutaric acid, TC) were able to discriminate completely each one of the three classes from the other two. Hence, to give a more comprehensive perspective of the results of the chemical analysis in association with the three different vinifications, multivariate statistical analysis was performed over the entire dataset (39 volatile compounds plus 11 phenols). Hierarchical cluster analysis using Euclidean distances and PCA were applied. A neat clustering of the three different winemaking procedures was obtained with HCA (Figure 5). The HCA dendrogram shows that the similarities between the two wooden containers were much higher than those between any wooden container and the amphorae. This difference may be also the result of the long maceration time between the wine and the solid parts of the berries (i.e., seed and skin) in the in-amphorae winemaking process (however, the discussion of the PCA reported below showed that the phenolic variables were not so effective in describing the sample variance as the volatile compounds were).

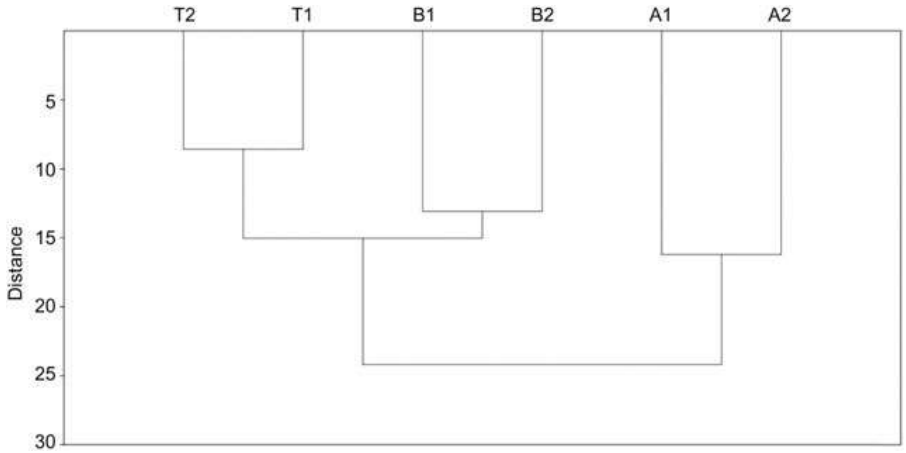



**Figure 5.** Hierarchical cluster analysis of the Chardonnay wines obtained in toasted barrels (B1 and B2), non toasted tanks (T2 and T2), and amphorae (A1 and A2).

Principal component analysis (Figure 6) offers a tool for visualizing the data structure by reducing the data dimensionality while retaining as much as possible the information present (Berrueta *et al.,* 2007). The PCA plot in Figure 6 (bi-plot) shows the loadings plot and scores plot in the space defined by the two first principal components PC-1 vs PC-2. The first two principal components using all the variables accounted for 68% of the total variance (PC-1, 46%; PC-2, 22%) with this model. The distribution of the samples and variables were strongly influenced by the storage conditions, since wine in non toasted tanks (T1 and T2) and toasted barrels (B1 and B2) were grouped in the upper right and in the lower right quadrants respectively, while in-amphorae samples were distributed in the central-left zone. Overall, the PC-1 differentiated successfully the wooden from the in-amphorae samples, whereas PC-2 differentiated effectively the large tanks from the toasted barrels. 1-Hexanol (17), limonene (24), 2-ethyl-1-hexanol (25), and linalool (31) correlated along PC-1 with in-amphorae samples (A). Ethyl propionate (8), ethyl hexanoate (23), ethyl 2-ethyl-hexanoate (26), ethyl 2-furancarboxylate (27), 2-nonanone (29), ethyl octanoate (35), ethyl decanoate (41), and ethyl dodecanoate (44) clustered closer to the non toasted wooden tank (T). Samples obtained in toasted oak containers (B) correlated instead with ethyl 2-methyl-propanoate (11), ethyl 2-methyl-butanoate (15), ethyl 3-methyl-butanoate (16), ionone (37), ethyl nonanoate (38), and sesquiterpene (43). Generally, the alcohols were clustered nearer to the in-amphorae samples (A1 and A2). Non branched ethyl esters clustered closer to the samples obtained in non toasted wooden

tanks (T1 and T2). Branched esters were clustered preferentially nearer to the wines obtained in toasted barrels (B1 and B2). Among the phenols, catechin (CA), caffeic acid (CF), p-coumaric acid (PC), epicatechin (EC), and protocatechuic acid (PR) were clustered closer to the in-amphorae wines. Gallic acid (GA), ethylcaffeate (ET), glutathionyl caftaric acid (GRP), caftaric acid (CT), cis-coutaric (CC), and trans-coutaric acid (TC) were positively correlated with wood containers. Notably, non toasted wooden tank wines (T1 and T2) contained a much higher amount of gallic acid compared with the other wines.

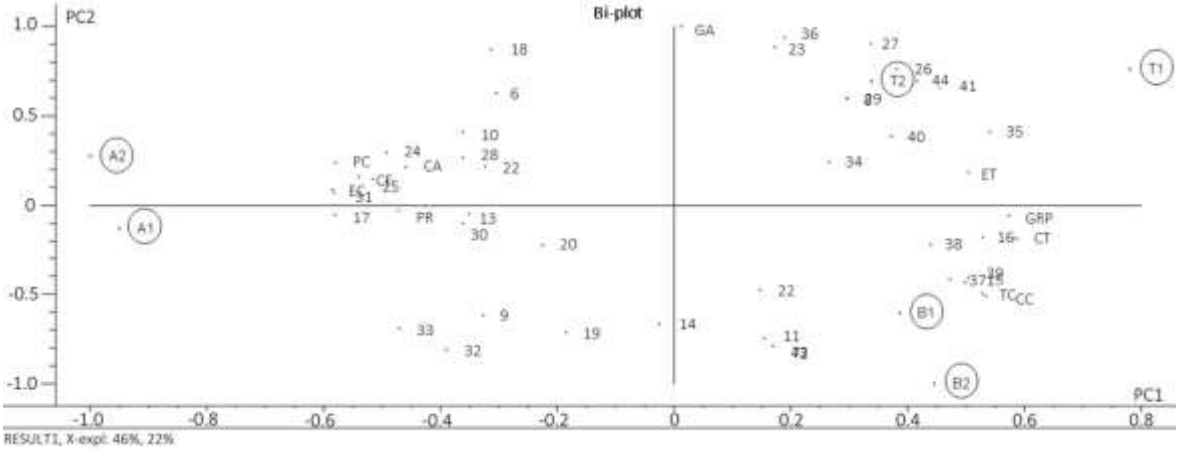



**Figure 6.** Principal components analysis of Chardonnay wines (PC 1 versus PC 2). A1 and A2, wines made in amphorae; T1 and T2, wines made in non toasted oak 2000-L tanks; B1 and B2, wines made in toasted oak 225-L barrels. **Volatile compounds**: 6 = propyl butanoate; 7 = acetic acid; 8 = ethyl propionate; 9 = 2-butyltetrahydrofuran; 10 = pantolactone; 11 = propanoic acid, 2-methyl-, ethyl ester; 12 = 2-pentanol; 13 = ethyl butanoate; 14 = propanoic acid, 2-hydroxy-; 15 = butanoic acid, 2-methyl-ethylester; 16 = butanoic acid, 3- methyl-ethylester; 17 = 1-hexanol; 18 = isoamyl acetate; 19 = pentyl acetate; 20 = furan, 2,2′-[oxybis (methylene)]bis-; 21 = 4-ethylbenzoic acid, 2-methylpropylester; 22 = n.i.; 23 = ethyl hexanoate; 24 = limonene; 25 = 2-ethyl-1-hexanol; 26 = ethyl 2-ethylhexanoate; 27 = ethyl 2- furancarboxylate; 28 = pentyl isobutyrate (amyl isobutyrate); 29 = 2-nonanone; 30 = terpinolene; 31 = linalool; 32 = phenylethanol; 33 = diethyl succinate; 34 = octanoic acid; 35 = ethyl octanoate; 36 = isopentyl hexanoate; 37 = ionone; 38 = ethyl nonanoate; 39 = naphthalene, 1,2-dihydro-1,1,6-trimethyl; 40 = 4-decenoic acid, ethylester; 41 = ethyl decanoate; 42 = octanoic acid, 3-methylbutylester; 43 = sesquiterpene; 44 = ethylester of dodecanoic acid. **Phenolic compounds**: GA, gallic acid; PR, protocatechuic acid; CT, caftaric acid; GRP, glutathionyl caftaric acid; CC, *cis*-coutaric acid; TC, *trans*-coutaric acid; CA, catechin; CF, caffeic acid; PC, *p*-coumaric acid; EC, epicatechin; ET, ethylcaffeate.

### *1.3.7 Sensory panel*

The sensory evaluation was less accurate than the chemical analysis in describing the samples variance. The radar plot in Figure 7 gives a schematic representation of the results. Due to the higher phenolic acids content (protocatechuic, p-coumaric, and caffeic), in-amphorae wines were characterized by a high pungent (AP) taste. Moreover, the presence of 2-ethyl-1-hexanol and 1-hexanol was possibly related in the in-amphorae wines to higher herbaceous/green (HG) and solvent (SA) characters, as already reported (Lee *et al.,* 2003; Mozzon *et al.,* 2016). Four variables could be used to differentiate in-amphorae wines compared with the other containers: solvent and acetone (SA), astringent/ pungency (AP), fruity (FR), and color intensity (CI). In particular, for CI, wines that scored 4.5 or less were made in amphorae, and the ones with 4.6 to 6.6 score were made in wood.

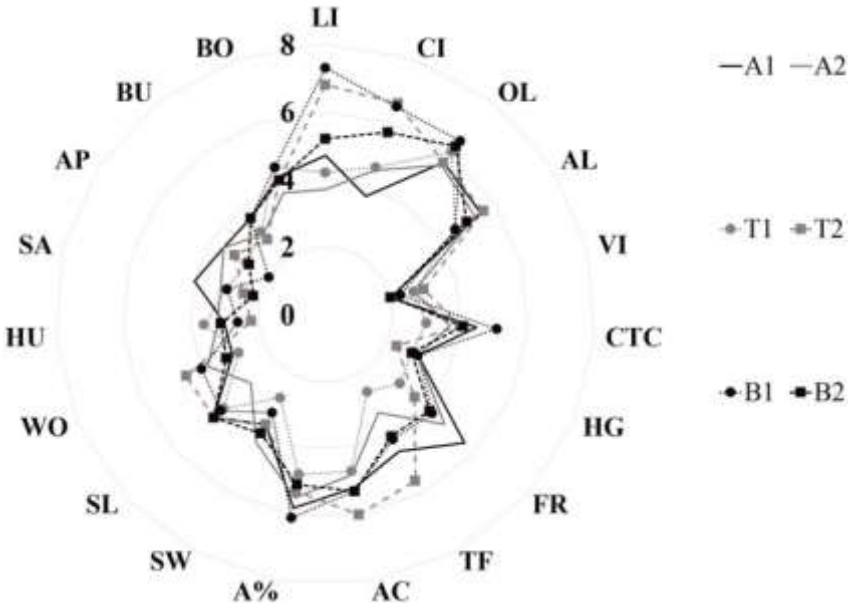



Figure 7. Sensory profile of Chardonnay wines obtained in toasted oak barrels (B1 and B2), non toasted oak tanks (T1 and T2), and in clay amphorae (A1 and A2). LI, limpidity; CI, color intensity; OL, olfactory intensity; AL, alcohol/liquor; VI, vinegar; CTC, caramel/toasted/ cookie; HG, herbaceous/green; FR, fruity; TF, tropical fruits; AC, acid/ citrus; A%, alcoholic; SW, sweet/honey; SL, salty; WO, wood/oak; HU, herbaceous/unripe; SA, solvent/acetone; AP, astringent/pungency; BU, burning; BO, wine "body" perception

## 1.4 Conclusion

Using a single variety of grape, such as Chardonnay, three chemically and sensorically different wines were obtained by using clay amphorae, non toasted wood barrels, and toasted barrique. The nature of the containers is considered to be a potential factor differentiating wines obtained from the same Chardonnay grapes. Sensory and chemical properties (enological parameters during the winemaking and volatile and phenolic composition after 1 year of bottle storage) were analyzed, and the data were processed by univariate and multivariate statistical analysis.

During six months of winemaking the main difference observed in amphorae with respect to barrels and barriques was the higher rate of conversion of malic acid into lactic acid, presumably due to the maceration with the pomace. The Chardonnay wine obtained in amphorae with maceration showed a remarkably higher final dry extract than the wine obtained from barrels and barriques. This was presumably due to the contact with the pomace, which led to the diffusion of extractable components. According to the trained panel, the amphorae wine resulted to have less sensory characters typical of the Chardonnay grape. Moreover, aromatic compounds typical of the aging in wood containers were not present in amphorae Chardonnay wine. The tannin content of this wine was appreciated, due to the maceration with the pomace, which was not related to the storage in oak wood.

Volatile profile and phenolic composition analyzed by mass spectrometry allowed classifying the Chardonnay wines obtained under different conditions after one year of bottle storage. Condensation reactions proceeding in the wood containers lead to esterification of linear (2000-l oak tanks) or branched (225-l toasted oak barrels) organic acids with ethanol and other alcohols. The in-amphorae wines were characterized by a higher contents of free phenolic acids and of higher volatile alcohols compared to wooden containers. The sensory and phenolic profiles were less effective than the volatiles in differentiating between earthenware and the wooden samples.

The results of this work provide a first insight on the chemical properties of a modern in-amphorae wine obtained from a grape variety of international importance.

Each container can influence the chemical and sensory quality of the final wine due to its peculiar geometry and material characteristics. By using the modern amphorae, winemakers may extend their commercial wine offer by exalting the characteristics of the grape in a different and innovative way, not related to the aging in wood.

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# CHAPTER 2

## The influence of the type of stopper and the bottle storage on phenolic and volatile composition of four Italian wines

## 2.1 Introduction

### *2.1.1 Wine evolution during the bottle storage*

Wine is an alcoholic beverage with peculiar chemical and organoleptic characteristics obtained at the end of the production process, before the bottling. The quality of wine is the result of several factors occurring from the grapes growing until the bottling and the storage of the final product: terroir, grape variety, maturity and sanitary conditions of the grapes, vinification technique, bottling and storage conditions affect the final expression of the wine attributes.

Between production and consumption, wine (especially red) spends a considerable part of its life cycle stored in bottle. Differently from most food products, which have their best quality level when fresh, wine may need an aging period to taste its optimum. In the case of red wine, the quality is expected to improve during the bottle storage period, the organoleptic characters are likely to evolve, the color to change, the aroma to develop in a more complex bouquet and the tannins to soften.

The time required to acquire the changing is very variable and strongly depends on the type of wine and its quality before bottling, however some premium red wines may need even decades (Ribéreau-Gayon *et al.*, 2006*)*.

The changes during the bottle storage, involve the chemical composition and the organoleptic properties: color, aroma, mouthfeel and taste evolve modifying the characteristics and the quality of the product (Skouroumounis *et al.*, 2005; Puskas *et al.*, 2012).

### *2.1.2 Influence of oxygen during the bottle storage*

The storage conditions (temperature, humidity, light exposure) affect the development of the wine (Boulton *et al.*, 1996). However, the foremost factors influencing its evolution are the chemical composition at the bottling time and the oxygen presence during the bottle aging period (Caillé *et al.*, 2010; Wirth *et al.*, 2010; Ugliano, 2013).

Considering the chemical composition, wine is a complex matrix rich in polyphenols and with a complex aroma. The phenolic profile in food and wine rises up to interest in consequence of their antioxidant properties and the related impact on human

health (Waterhouse *et al.,* 2006). The main type of phenolic compounds present in grapes are represented by anthocyanins, present in skins only, hydroxycinnamic acids, abundant in pulp and skins, and flavanols, that include monomers (catechins), oligomers and polymers (called proanthocyanidins, or condensed tannins), localized in seeds, skins and in smaller amounts in pulp (Mane *et al.,* 2007).

One of the most attractive and controversial topic of debate on wine concerns its aroma. The aromatic profile is a complex interaction of a wide range of molecules. Some research identified up to 1000 compounds but only several tens of them can be perceived by humans (Tao *et al.*, 2009). Most of the aroma compounds are present in the volatile fraction and they belong to the chemical classes of alcohols, aldehydes, esters, acids, monoterpenes and other minor compounds. The aromatic characteristics of young wine is primarily made of fruity and flowers aroma that derived from fermentation. During aging, wine aroma tends to evolve in the appearance of the so-called developed characters as result of the occurrence of numerous reactions.

Considering the second parameter, the bottle is not an inert system and oxygen can enter the bottle in different quantities depending on the different types of closures (Lopes *et al.,* 2006; 2007). The parameter defining the amount of oxygen permeating through a material is called Oxygen Transmission Rate (OTR) and it is often applied to assessing wine closures (Dieval *et al.*, 2011).

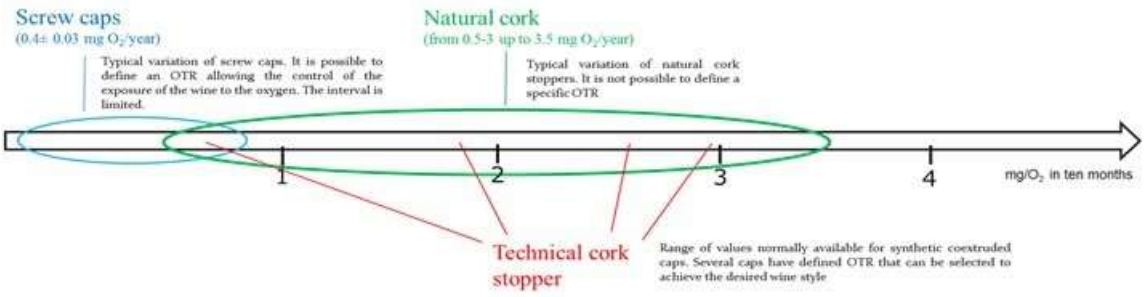


**Figure 8**. Oxygen Transfer Rate for different types of stopper (mg $O_2$ / year) (Figure adapted from Nomacorc Informativa tecnica 04; OTR e profili aromatici dei vini rossi).

Godden *et al.* (2001) demonstrated the influence of the sealing system and the OTR on the evolution of wine during the bottle storage. However, it may be misleading to consider the OTR as the only parameter for measuring the level of oxygen into the bottle. In fact, during winemaking oxygenation occurs in significant way thus, a

certain amount of oxygen dissolved in wine before bottling (Dissolved Oxygen). Moreover, air can enter the bottle through the interface between the stopper and the bottle. In addition, the stopper itself can release oxygen after the compression at bottling, a phenomenon known as outgassing (Figure 9).

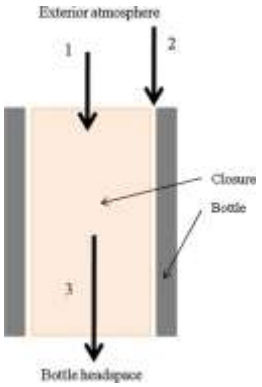


**Figure. 9.** Oxygen paths for entering the bottle during wine ageing. (1) Diffusion through closure's pores; (2) Diffusion in the interface between the closure and the bottle; (3) Oxygen expelled from the closure during compression at bottling (Silva *et al.,* 2011).

Definitely, since bottling, the total amount of oxygen present in the bottle is referred to as total package oxygen (TPO). This is considered as the sum of the dissolved oxygen (DO) in wine and the oxygen concentration in the headspace of the bottle (HSO). The specific focus on the oxygen during the wine storage is due its ability to strongly interact with certain wine components such as, ethanol, phenolic, volatile compounds and sulphur-containing natural compounds (Escribano-Bailón *et al.*, 2001; Waterhouse *et al.,* 2006). The impact of oxygen on phenolic composition, color, aroma or sensory characteristic of bottle-aged wine were largely investigated (Hopfer *et al.*, 2013; Saenz-Navajas *et al.*, 2014; Liu *et al.,* 2016). Phenolics are known to be an optimum substrate of reaction with oxygen due to their hydrogen-donating attitude (Wildenradt, 1974) but also between themselves. Anthocyanins and flavanols bind together through cross-linking reactions giving origin to flavanol-anthocyanin adducts (Somers, 1971). Consequently, their condensation with the acetaldehyde results into flavanol-methylmethine–anthocyanins adducts (through methylmethine bridge often referred as to ethyl-bond) and pyranoanthocyanins-flavanol adducts (Wirth *et al.,* 2012).

Acetaldehyde is the main aldehyde in wine and it derives from the oxidation of ethanol (Silva *et al.,* 2011). In minor part, acetaldehydes is a product of the microbial

activity. Both yeasts and acetic acid bacteria are able to produce aldehydes respectively as an alcoholic fermentation leakage product and as the oxidation product of ethanol, with the second one having the highest production (Liu *et al.,* 2000).

At low concentration, acetaldehydes is responsible of pleasant fruity aroma, whereas at higher level it possesses an irritating and pungent aroma (green, grassy or apple-like off-flavour) (Henschke *et al.*, 1993).

### *2.1.3 Cork Stopper*

Cork is a material obtained from the bark of a tree, the Cork oak (*Quercus suber L.*). Generally, it is removed every 9-12 years to assure the cork layer reached the minimum required thickness (Silva *et al.,* 2005). The stripping does not harm the tree and the bark regrows. The first extraction takes place when the tree has reached a circumference of 60 cm at a height of 130 cm. However, the material obtained from the first collection, called "virgin cork" is of little value, not usable for the production of one-piece stopper. Only from the second extraction, which must be carried out at least ten years after the first, is obtained the cork known as "female or gentle cork", of better quality. The third stripping is suitable for manufacture cork stoppers. The cork is a material with a very low density (0.20 g/cm$^3$) with a characteristic cellular structure in which the cells are ordered in regular planes. The cellular wall is made of cellulose on the inside, of lignin on the outside and a dominant layer of suberine in the intermediate part. The latter, is the organic substance that confers the properties making cork ideal for closing wine bottles: resistance, almost total inertia, impermeability, plasticity, flexibility, adhesion, combined with easy processing and considerable duration. The consistent presence of soluble tannins in fresh cork, which would be easily yielded to the wine, is removed through depuration steps and multiple boiling of the planks (previously seasoned from 6 to 24 months) during the industrial processing. Then, the planks are dried and stored for some weeks at controlled temperature and relative humidity to be stabilized. Depending on the quality (structural defects and porosity) and on the thickness, the planks are ranked in up to seven categories (Borges *et al.,* 1985; Pereira *et al.*, 1994). Natural stoppers are punched from the best planks manually or automatically. The remaining material

resulting from extraction will be used for the production of agglomerate stoppers, disks, or other types of cork agglomerates (Borges *et al.,* 1985; Silva *et al*., 2005). Stoppers are sterilized in special solutions, dried to bring the moisture content to 6-8% and lubricated. This operation is necessary to facilitate the entry of the cork into the neck of the bottle and to reduce the powder formation. Traditionally, it consisted in coating with paraffin but today it has been replaced by chemically inert food-grade silicones. The production process does not prevent the "cork taint" from being transmitted to the wine, which irreparably damages the content of the bottle. 2,4,6-Trichloroanisole (TCA) was identified in the early 1980s as a cause of cork taint (Buser *et al*. 1982) but recent research have found that also 2,3,4,6-tetrachloroanisole (2,3,4,6-TeCA) and pentachloroanisole (PCA) can contribute to a lesser extent (Butzke, 1999). TCA is an exogenous compound to wood, wine and cork stoppers. When any of these materials containing phenols enter in contact with a chlorinated substance the trichlorophenols emerge. For example, chlorophenols can derive from chlorine bleaching process applied to sterilize or bleach wood, paper, and other materials. They can easily migrate from the atmosphere, water or other objects such as shipping pallets treated by chlorophenols into glass bottles, barrels and cork stoppers. Subsequently, the nontoxic anisoles are thought to arise by O-methylation of the highly toxic chlorophenol precursors, as part of a normal detoxification reaction mediated by different microbial species (Cserjesi *et al.,* 1972; Allard *et al.,* 1987; Neilson *et al.,* 1988). Considering the biodiversity of the environment where cork oaks grow and the various steps of the manufacturing process hitherto has not been possible to unequivocally identify the microorganisms responsible for the appearance of 2,4,6-TCA and the biochemical pathways or mechanisms leading to its formation (Alvarez-Rodrıguez *et al.*, 2002).

Historically, the cork was used since the Romans and the Greece to close jars containing food and beverages. It was appreciated for its characteristics namely, ability to retain air, elasticity, ease of removal (Kontoudakis *et al.*, 2008). Since the 18$^{th}$ century, the cork was the only material used for the wine closure. However, stoppers made of cork have some weaknesses, due to the heterogeneity of its structure. The production is difficult to standardize: low quality corks may cause the diffusion of excessive gas amounts (OTR in cork stopper is highly variable (Godden

*et al.,* 2005; Limmer, 2006)) and the risk of cork taint, provoking serious defects to the wine (Mas *et al.*, 2002). Since the nineties, the demand of a high-quality wine with reproducible characteristics and the competition between the wine producers pushed for the search of alternative type of closures. This is the case of caps resembling those of cork but made of synthetic materials or composed of a mixture of synthetic and natural materials (technical stoppers). One of the most recent type of alternative stoppers are those entirely made of microgranule cork without glue addition. The advantage of the alternative stoppers is the prediction of a constant OTR (Ferreira *et al.,* 2003) compared to the variability of the cork one.

### *2.1.4 Alternative type of stoppers*

Nowadays the market offers several options beside the classic one-piece natural cork stopper.

(1) Colmated stopper is a one-piece natural cork where lenticels are filled with a surface treatment using cork powder associated with FDA-approved natural resin glues or water-based glues. It aims to eliminate surface imperfections of the cork and to improve mechanical and visual properties.

(2) Multi-piece natural cork consists of two or more pieces of cork joined by a glue. Generally, the cork comes from slow-growing, low-thickness planks, which gives the cap a high density.

(3) Agglomerated stoppers referred to as technical stoppers, consist of microgranule cork (2-8 mm sizes) obtained by grinding and / or crushing and classified by grain size and by density.

(4) Agglomerated stoppers with discs, differentiated in 1 + 1 or 0 + 1 or 0 + 2 depending on the number of discs and their position relative to the two sides of the cap. These characteristics provide a closure that is chemically stable and mechanically strong.

(5) Next-generation agglomerate stoppers are obtained by agglutination of cork granules treated with or without adhesives and composed of at least 75% of cork granules (by weight).

(6) Synthetic closures are made from a high-grade thermoplastic elastomers (TPE), a class of polymeric mixture (usually plastic and rubber) that contributes to confer the elastic properties. They have the main advantage of not to have lenticels which can harbor bacteria in the cork stoppers.

(7) Screw caps refer to any metal cap which is applied over a bottle top. The metal screw cap with the appropriate liner is a barrier which air cannot diffuse through. The term Stelvin is used nowadays interchangeably but should specifically refer to the patented closure of Pechiney, France and the term Savin cap to refer to the product of MCG Industries in South Africa (Shinde *et al.,* 2016).

So far, investigations on the influence of the type of stopper concerned the evolution of the phenolic content during the bottle storage (Gao *et al.* 2015; Xing *et al.*, 2016) and in relation to the oxygen content at bottling (Saenz-Navajas *et al.*, 2014). Other studies outlined the relationship between the evolution of the volatile profile in bottle-aged wines (Ugliano, 2013) and the use of different types of stoppers (Liu *et al.*, 2016). In this study, the phenolic and volatile profiles of three red and one rosé wine from South Tyrol region (Northern Italy) were monitored regularly over a bottle storage period of twelve months at specific time steps. Herein, the aim was to investigate the influence of the type of stopper and the bottle storage time on the evolution of the phenolic and volatile compounds. This work would provide further knowledge in preserving the wine quality attributes during the bottle storage period.

## 2.2 Material and methods

### *2.2.1 Wine Samples*

The selected wine samples were four Italian wines provided by Kellerei Bozen winery (Gries, Bolzano) in the South Tyrol region (Northern Italy): Merlot, Lagrein red, Lagrein rosé and Santa Magdalener wine. Only the St. Magdalener wine is a blend obtained from Schiava (dominant) and Lagrein grape cultivars whereas, Merlot, Lagrein red and Lagrein rosé were obtained only from the corresponding grapes cultivar (monovarietal). All the grape varieties were harvested in the vintage of 2016

from vineyards located in the South Tyrol area; the wines obtained were bottled the following year (summer 2017). Kellerei Bozen winery provided twenty bottles for each type of wine, ten closed with the Supercap Nature stopper and ten with the conventional stopper. The bottles were stored horizontally at room temperature and the analysis were performed during the storage period at five specific times: at the bottling time ($0^{th}$ month) and at the $1^{st}$, $3^{rd}$, $6^{th}$, and $12^{th}$ month after bottling. At each sampling, four bottles for each type of wine (closed with the two different stoppers) were analyzed.

### *2.2.2 Stoppers*

The Italian company Supercap Srl (Pesaro e Urbino, Italy) provided the Supercap Nature stoppers (Figure 10). This is a new generation technical cork stopper composed of a sanitized cork micro-granules blend with high-performance polymers without glue addition. The stoppers used for the comparative analysis included two "1+1 technical" cork stoppers, one whole-piece natural cork and one agglomerated cork stopper (Figure 11).

**Figure 10**. Supercap stopper

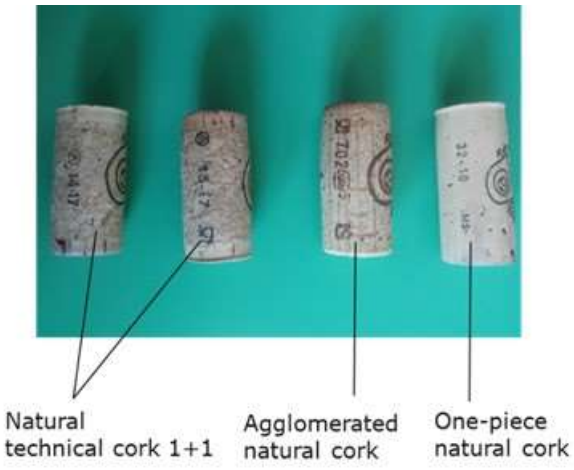


**Figure 11.** Stoppers used for the comparative analysis with the Supercap stopper.

### *2.2.3 Chemicals and reagents*

All reagents used in this study were purchased from Sigma-Aldrich S.p.A. (Milan, Italy) whereas the solvents (analytical grade) were obtained from VWR International s.r.l. (Milan, Italy).

### *2.2.4 Non-anthocyanin phenolic compounds determination*

The analysis of the non-anthocyanin polyphenols and the cyclic proanthocyanidins were performed through a UHPLC-HRMS system (Agilent 1290 Infinity) equipped with a UV-Vis diode array detector (DAD) (1290 Infinity) connected to a quadrupole time of flight mass spectrometer (QTof/MS) with an electrospray ionization source (ESI) source (Agilent 6530 Agilent Accurate Mass). Figure 12 reported a UV chromatogram of a Merlot sample. The UHPLC-HRMS analyses were carried out on a C18 reversed phase column (2.1 × 100 mm, 1.8 µm, Agilent). Samples were directly filtered on a 0.45 µm membrane filter before the injection. The temperature of the column was at 25°C and the flow rate was set at 0.3 ml/min with the injection set at 2.0 µl. The mobile phases consisted of (A) aqueous 0.1% formic acid and (B) methanol 0.1% formic acid. For non-anthocyanin polyphenols (phenolic acids, flavan-3-ols, stilbens), the gradient of solvent B was as follows: 6% for 0.5 min; 6 to 40% for 29.5 min; 40 to 100% for 8 min; 100% for 5 min; 100 to 6% for 2 min. followed by washing and re-equilibration for 3 min. The mass spectrometer was operated in extended dynamic range of 2 GHz (m/z 3200). The nebulizer pressure and flow rate were set at 25 psi and 9 l/min, respectively. Its drying gas temperature was 300°C. The sheath gas flow and temperature were set at 11 l/min and 350°C. The fragmentation, skimmer, OCT and capillary voltage were at 150 V, 65 V, 750 V and 4000 V, respectively. The analyses were performed in negative ionization mode. The data analysis was performed on Mass Hunter Qualitative Analysis software. Phenolic compounds were identified by comparing their chromatographic retention times and accurate masses with those of pure standard compounds. The calibration curves of pure standard substances were established through the DAD and were used to quantify the phenolic concentrations. When reference compounds were not available, a calibration with structurally related standard substances was used (gallic

acid for protocatechuic acid and syringic acid; caffeic acid for caftaric acid, and glutathionyl caftaric acid [GRP]; (+)-catechin for (-)-epicatechin. The integration of the peaks allowed obtaining the concentrations of the identified compounds. Concentrations were expressed in mg/l of standard or of the structurally related standard. Absorbance values of the cyclic proanthocyanidins were registered for evaluating their abundances.

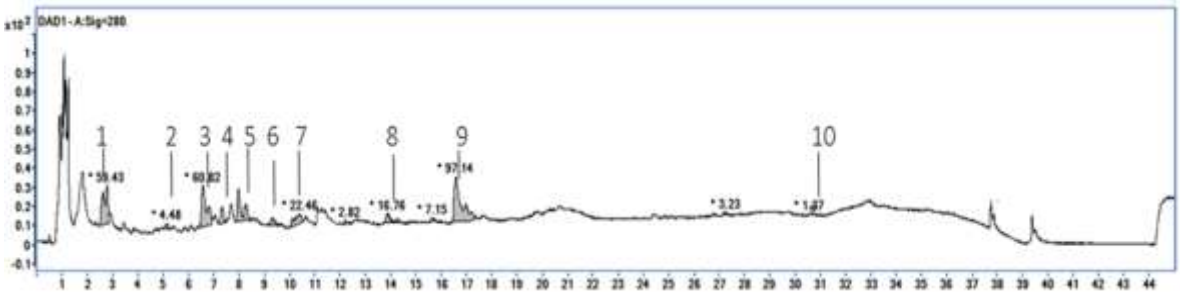


**Figure 12.** UV chromatogram (λ = 280 nm) of a Merlot sample wine with the identified peaks indicated and numbered. Peak assignments are reported in Table 5.

### *2.2.5 Anthocyanins determination*

Determination of relative composition for three anthocyanins classes (glucoside, acetyl-glucoside and coumaroyl-glucoside) was evaluated using the official method adopted by OIV (Vines and Wines International Organization). Samples were directly filtered on a 0.45 µm membrane filter before the analysis. The HPLC system Accela series (Thermo-Scientific, Illkirch-Graffenstaden, France) was equipped with a 4 × 250 mm internal diameter (i.d.), 5 µm Nucleosil C18 column (Agilent, Les Ulis, France). The solvents used were water (Eluent A) and acetonitrile (Eluent B) both containing 5% of formic acid. The gradient of solvent B consisted of 10% – 23% in 16 min, 23% – 28% in 19 min, 28% – 100% in 6 min at a flow rate of 1 ml/min. The column was washed with 100% acetonitrile for 5 minutes and re-equilibrated with the initial conditions for 3 minutes. The peaks identification was performed in accordance with bibliography (Chira, 2009). The quantification was carried out through the injection of external standard of malvidin-3-*O*-glucoside (Mv3G).

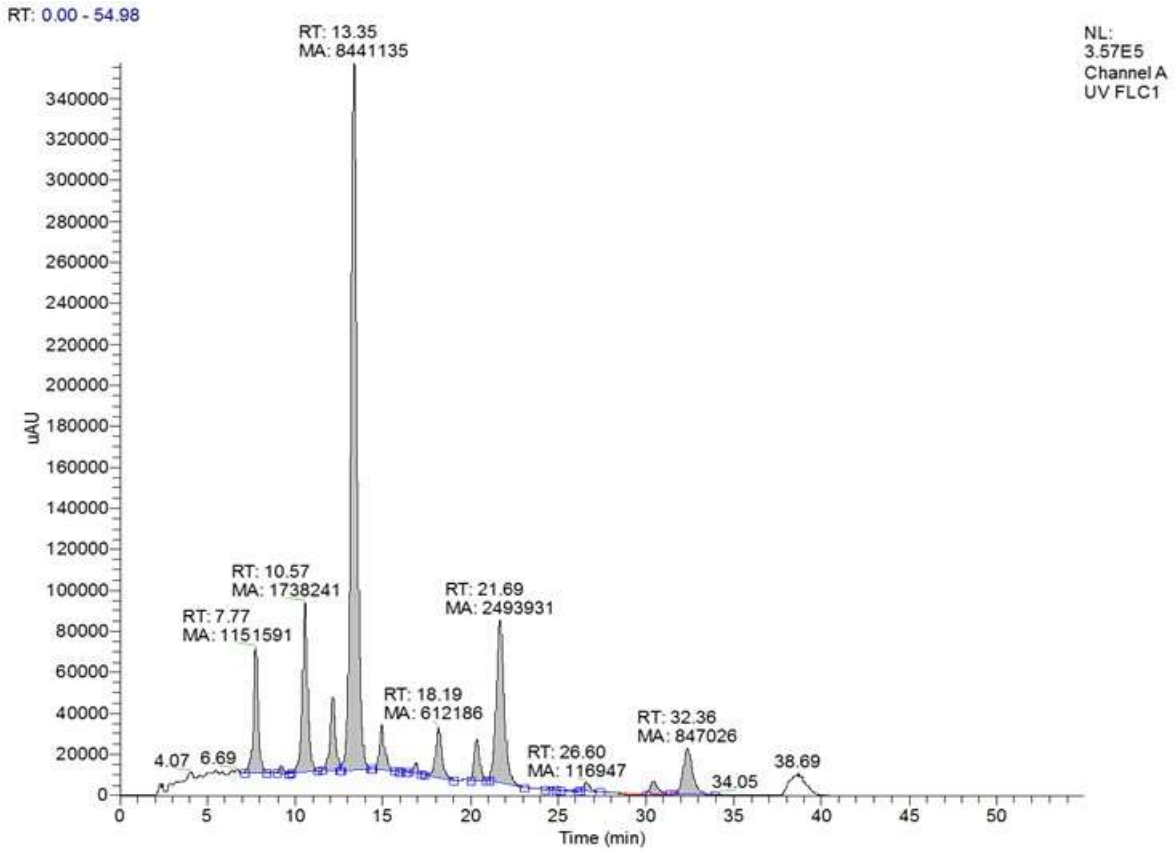


**Figure 13**. UV chromatogram (λ = 520 nm) of a Merlot sample wine with the identified peaks indicated.

### *2.2.6 Volatile compounds determination*

Volatile profile was obtained through gas-chromatography mass-spectrometry (GC-MS) after extraction with head-space solid-phase micro-extraction (HS-SPME). An example of a chromatogram obtained is shown in Figure 14. Volatile compounds determination was performed according to a published procedure (Rodrigues *et al.,* 2008), with slight modifications. Briefly, 10 ml of wine were introduced into a 20-mL vial, blended with 1 g NaCl and tightly capped with a screw cap equipped with an elastomeric septum. The vial was equilibrated in a heating bath at 40°C for 10 min. Afterwards, a SPME fiber coated with 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; Supelco/Sigma-Aldrich, (Milan, Italy) was inserted into the vial and exposed to the sample headspace for 20 minutes under continuous heating. Subsequently, the thermal desorption took place in the GC injector at 220°C for 3 minutes. A Varian 3900 gas-chromatograph coupled to a Saturn 2100T (Varian, Walnut Creek, CA, USA) ion trap mass

spectrometer was equipped with a ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm I.D., film thickness 0.25 μm). The injection was in splitless mode (splitless time 0.3 min) and the temperature program of the GC oven was conducted as follow: holding 40 °C for 10 min, then raising to 180 °C at a rate of 3 °C/min and reached 250 °C at 15 °C/min. The MS transfer line and trap temperatures were set at 200 °C. The ion trap emission current was 10 μA. The mass spectra were recorded in the full scan mode (mass range 31-250 m/z) at 1 scan/sec. Data were analysed with the Varian Workstation software. Tentative identification was based on the comparison with the NIST library mass spectra (Version: 2.0; 2002), the GC linear retention indices reported in the literature and through the injection of pure standard substances when available. Samples were analysed in duplicate (two different bottles). Quantification of the peaks area was expressed as internal area percentage.

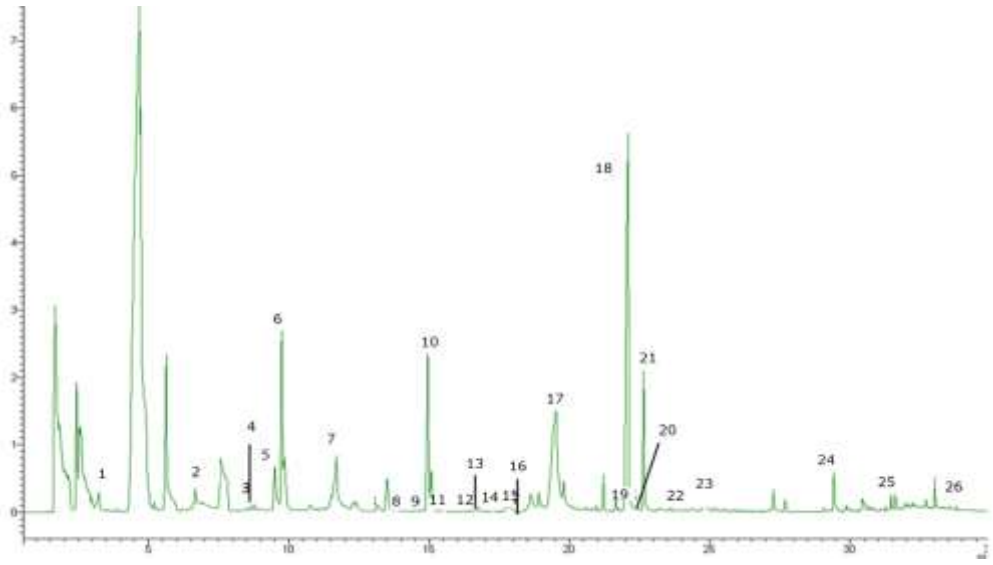


**Figure 14**. Chromatogram of a Merlot sample obtained through GC.
Corresponding peak numbers are reported in Table 7.

### *2.2.7 Dissolved oxygen content*

The dissolved oxygen content was measured through the L.sensor-700.02 device provided by FT system. The analysis were performed by the laboratory staff of the Supercap Srl company (Pesaro e Urbino, Italy).

### *2.2.8 Sensory analysis*

The discriminant triangle test was chosen for evaluating the possible differences between the wines closed with the different stoppers (ISO 4120: 2007). Each session was repeated on two consecutive days. Sensory analyzes were conducted in two different locations: at the enology research unit of the Institut de Science de la Vigne et du Vin (ISVV, Bordeaux, France) for the tasting sessions at bottling time (T1), after one (T2) and three months (T3) from bottling. The panel consisted of professional wine judges from the same research unit (ISVV, France). There w ere ten women and eight men, aged between 23 and 60 years. Whereas, the tasting session carried out at six (T4) and twelve months (T5) from bottling were performed at the Faculty of Science and Technology of the Free University of Bozen. In this case, judges were PhD students and researchers of the food science faculty aged between 24 and 57 years. All judges were asked to taste three glasses of wine prepared in each booth and asked to select the one they felt different. The room were designed to limit external factors that could potentially disturb sensory analysis and corresponds to the AFNOR (ISO 8589) standards for this type of equipment (sound insulation, constantly regulated temperature ,etc.). In all experiments, glasses were labeled with three-digit random codes and presented to the panelists according to the order provided by the standard methodolog (ISO 4120: 2007).

### *2.2.9 Statistical Analysis*

All data obtained on the chemical compounds by the instrumental analysis were expressed as mean of the measurements performed on duplicate bottles. Two-way analysis of variance (two-way ANOVA) with the storage time and stopper type was performed using GraphPad Prism v 6.01 software (San Diego, CA, USA). When significant differences were revealed ($p < 0.05$), Tukey (HSD) multiple comparison test was applied to compare the mean concentrations.

Multivariate statistics was applied with normalized data of the phenolic and volatile concentrations. Hierarchical Cluster Analysis (HCA) was performed to assess the similarities between the wines employing Euclidean distance and Ward's linkage

method. Principal Component Analysis (PCA) was carried out to confirm the HCA and to evaluate the influence of phenols and volatiles on the wines closed with the different stoppers. PCA and HCA were both performed using the PAST software V 3.18 (Hammer & Harper).

## 2.3 Results and Discussion

### *2.3.1 Phenolic compounds evolution during the bottle storage*

During one year of bottle storage the four wines were sampled at 5 specific time steps: bottling time (T1), after one month (T2), three months (T3), six months (T4) and after twelve months (T5). The analysis of the phenolic profile allowed the identification of ten non-anthocyanin phenolic compounds. Moreover, the anthocyanins detected were those typically present in wine grapes obtained from *Vitis vinifera* L. cv., derivative of delphinidin, cyanidin, petunidin, peonidin and malvidin. They were grouped in three families according to the esterification groups: glucoside, acetyl-glucoside and cumaroyl-glucoside derivates. Novel cyclic proanthocyanidins (tetramer and pentamer, described in detail in Chapter 3) were also identified.
The phenolic concentration means and standard deviations are reported in Table 5 and Table 6 for non-anthocyanins and anthocyanins respectively, whereas the cyclic proanthocyanidins are reported as mean of the absorbance detected through UV-vis DAD (Table 4.).

*Non-Anthocyanins compounds*

Each of the four wines had a different phenolic concentration due to the natural composition of the grape varieties used. However, a common evolution trend was detected for six out of ten non-anthocyanin compounds, namely gallic acid, caffeic acid, *p*-coumaric acid, *trans*-resveratrol, GRP and protocatechuic acid. During the first three months of bottle storage (T1, T2, T3) the concentrations remained constant (Figure 16-19). At six months (T4) a net increase followed by a clear reduction at 12 months (T5) was observed. This trend was detected in the three red wines. In Lagrein rosé wine this evolution trend was verified only for gallic acid. The other compounds did not show a mutual evolution trend. A comparison of the final concentrations (T5)

with the initial ones (T1) showed a reduction typical of the low molecular weight compounds such as caffeic acid, (+)-catechin, (-)-epicatechin and *p*-coumaric already reported in previous studies (Castellari *et al.,* 2000) and detected also after longer bottle storage period (18 months) by Gao *et al.* (2015).

*Anthocyanins compounds*

Considering anthocyanins at bottling time, the Lagrein red wine had the highest concentration compared to Merlot and Santa Magdalener (Table 6). Non-acylated anthocyanins in Lagrein red wine reported values of ~265 mg/l compared with ~160 mg/l for Merlot and ~142 mg/l for Santa Magdalener. In addition, acetylated and coumaroylated anthocyanins were also present in major concentrations in Lagrein red (~86 mg/l and ~27 mg/l) followed by Merlot (~48 and ~19 mg/l) and Santa Magdalener (~29 and ~13 mg/l). Rosé wine had predictably much lower concentration of anthocyanins (non-acylated ~28 mg/l, acetylated ~14 mg/l and coumaroylated ~7 mg/l) compared to the three red wines, due to the vinification process that had a shorter period of contact with the berries' skin. Nonetheless, in all four wines the sum of the three classes and thus the total amount of identified anthocyanins showed a clear reduction over the storage period (Figure 20-23). The reduction of the phenolic content during the bottle storage is generally ascribed to polymerization, oxidation and complexation reactions (Cheynier *et al.*, 1990; Gómez-Plaza *et al.*, 2002; Zafrilla *et al.*, 2003). In this case the dissolved oxygen of the four wines was low since the bottling (0.4-1.5 mg/l in red and 3.0 mg/l in rosé wine) and varied slightly during six months of storage. The low level of initial oxygen concentration may suggest that the reduction of the polyphenols resulted from polymerization reactions such as flavanol-anthocyanin complexation rather than from oxygen-mediated reactions.

*Cyclic Proanthocyanidins Tetramer and Pentamer*

Recent investigations on proanthocyanidins in wine reported the detection of a novel class with a cyclic structure, named crown tannins (Jourdes *et al.,* 2016; Jouin *et al.,* 2017). In this work, the investigation of these molecules allowed the identification of cyclic tetramer and pentamer proanthocyanidins. The analyzes monitored their abundance during three months of storage in bottle and their value were expressed in absorbance (Table 4). In each wine, tetramers resulted more abundant than

pentamers; Santa Magdalener and Lagrein red wines reported the highest abundance during three months of storage whereas, Lagrein rosé had the lowest value.

**Table 4**. Abundance of Cyclic Tetramer and Pentamer during three months of storage in bottle (T1, T2, T3) expressed in Absorbance (mean ± st. dev.).

| | | **Tetramer** | | **Pentamer** | |
|---|---|---|---|---|---|
| | | Conventional | Supercap | Conventional | Supercap |
| Merlot | $T_1$ | 43866.1±2618.7 | 45765.7±369.7 | 24103.1±402.3 | 23927.4±620.5 |
| | $T_2$ | 42411±6281.2 | 36826.4±3458.3 | 19464.4±394.3 | 20970.7±1888.5 |
| | $T_3$ | 56806.6±1708.7 | 60353.4±8586.9 | 23519.7±727.7 | 22111.1±3697.6 |
| Lagrein rosé | $T_1$ | 14481.4±492.1 | 12062.4±381.2 | 7207±289.1 | 6971.9±762.9 |
| | $T_2$ | 13989.8±96.7 | 12137.3±1392.9 | 8446.3±570.4 | 8028.9±2429.6 |
| | $T_3$ | 20821.1±1078.5 | 7328.8±921.3 | 20790.8±643.7 | 6639.5±333.5 |
| St.Magda. | $T_1$ | 64715.9±416.7 | 72943.5±3589.1 | 43361.6±478.9 | 42539.1±2193.7 |
| | $T_2$ | 97191.7±3678.2 | 103197.9±982.4 | 69230±4285.3 | 76974.3±2494.6 |
| | $T_3$ | 88999.5±885.4 | 84540.6±269 | 44137.5±210.8 | 43359.8±453.8 |
| Lagrein red | $T_1$ | 60964.2±1968.4 | 58346.6±1165.9 | 35960.7±996.7 | 36254.1±1756.1 |
| | $T_2$ | 68801.1±3092.3 | 61466.5±11986.3 | 42129.8±1443.7 | 37185.6±10046.1 |
| | $T_3$ | 88423.9±4394.2 | 82505.5±4953.1 | 40433.4±1362.5 | 39615.3±3951.7 |

Cyclic tetramer reported a similar trend in Merlot, Lagrein red and Lagrein rosé with a visible increase between the first (T2) and the third month of storage (T3) (Figure 15). Whereas, in Santa Magdalener the abundance reached the maximum in the first month of storage and then decreased until the third month. This trend was the same detected for the pentamer in Santa Magdalener wine. Whereas, in Merlot, Lagrein red and Lagrein rosé, the abundance of pentamers remained constant during the monitored period. Comparing the two types of stoppers, each wine showed a similar trend during three months of storage. Statistic T-test did not report any significant difference between the samples closed with the two stoppers at each sampling time (T1, T2, T3).

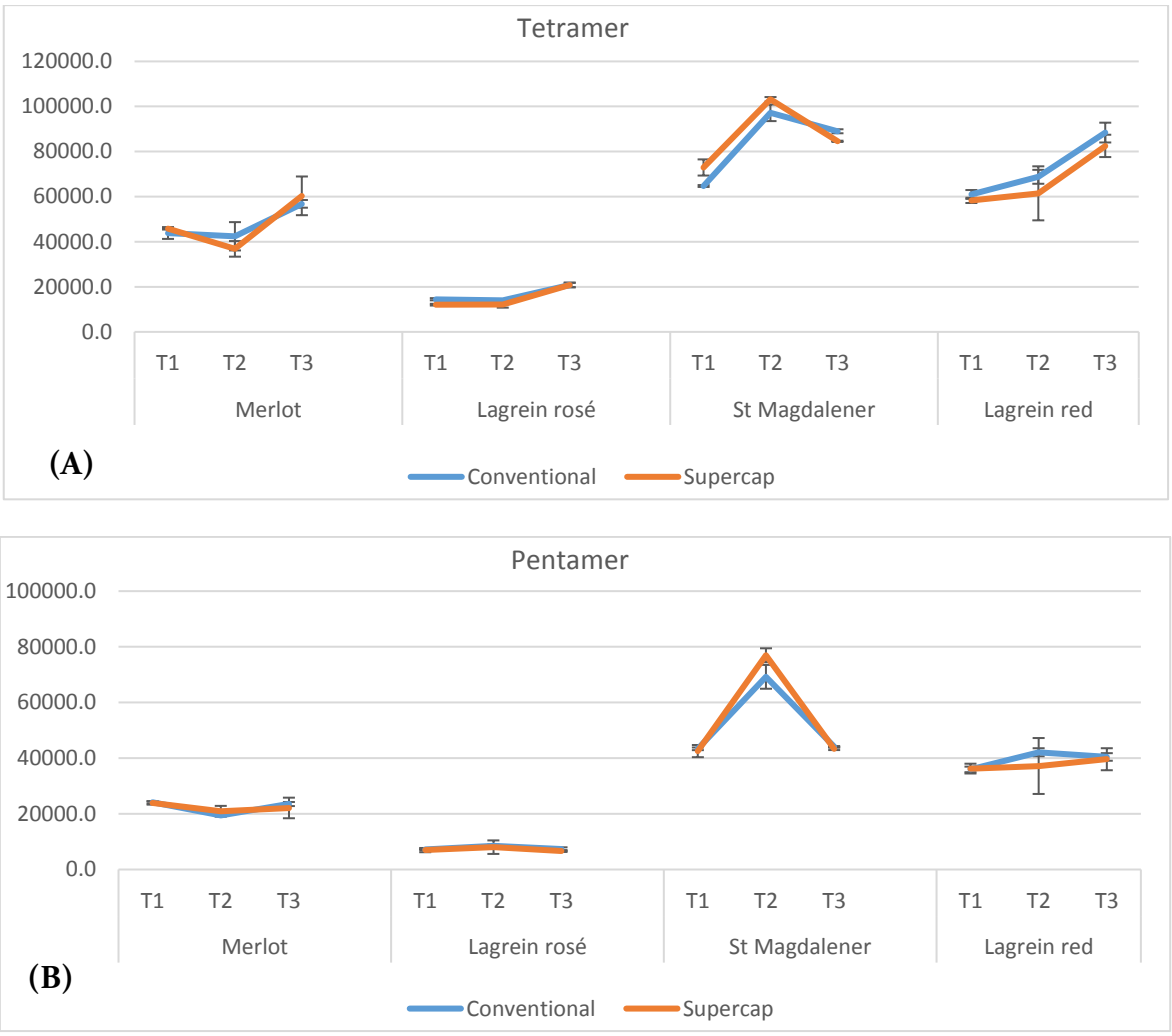


**Figure 15.** Abundance of Cyclic Tetramer **(A)** and Pentamer **(B)** during three months of storage in bottle (T1, T2, T3) expressed in Absorbance (mean ± st. dev).

### *2.3.2 Effect of the type of stopper and storage time on the phenolic composition*

The two-way ANOVA was performed to assess the influence of the type of stopper and the bottle storage time on the phenolic composition of the four wines (cyclic proanthocyanidins were not included in this analysis). The results showed that the storage time (*F storage period*) significantly influenced the phenolic concentration (Table 5 and Table 6). Notably, GRP, glucoside anthocyanins, acetyl-glucoside and cumaroyl-glucoside anthocyanins in Lagrein rosé ($F$ = 8570; 34170; 20523; 10927, respectively), caffeic acid, GRP and glucoside anthocyanins in Merlot ($F$ = 3435; 3263; 3439, respectively) showed high value for $F$ parameter related to the storage period. Moreover, GRP, glucoside anthocyanins and acetyl-glucoside anthocyanins in Santa Magdalener ($F$ = 2861; 4767; 2971, respectively), and p-coumaric acid in Lagrein ($F$ =

4297) were also strongly affected by the storage period. Whereas, the influence of the type of stopper at each sampling time (*F stopper*) affected significantly the phenolic compounds only in few cases. The wines closed with the Supercap Nature stopper reported lower significant concentrations with respect to the samples closed with the conventional stopper in Lagrein red wine for glucoside and acetyl glucoside anthocyanins, (+)-catechin and caffeic acid at 3 and 6 months respectively and for *p*-coumaric acid at 1 month after bottling (T2). Merlot wines reported differences for acetyl-glucoside anthocyanins at bottling (T1) and after 1 (T2) and 3 months (T3), but these times higher concentrations were detected in Supercap Nature samples. Moreover, Lagrein rosé showed a higher abundance of *p*-coumaric acid at T2 in wine sample closed with the Supercap Nature stopper. St. Magdalener wines differed for the lower concentrations in the Supercap Nature samples for GRP, glucoside anthocyanins and acetyl-glucoside anthocyanins at 6 months after bottling (T4).

These results showed a large variability of the statistical significance between the two types of stoppers. In fact, considering the four wines, it was not highlighted a common trend that could relate the type of stopper with the phenolic compounds. Besides, the statistical results highlight the dominant influence on the phenolic composition of the storage time over the type of stopper. In contrast, other studies demonstrated that different OTR of the stoppers effectively affected the phenolic composition during the bottle storage (Wirth *et al.,* 2010). However, in this study the statistical comparison of closures regarded each step of time individually (*F Stopper* at T1, T2, T3, T4, T5). This specific assessment aimed at evaluating the type of closure as unique parameter in a precise period of the storage in bottle.

Lagrein red


**Figure 16.** Non-anthocyanin concentrations (mg/l) in Lagrein red wines at the sampling times: T1, T2, T3, T4, T5 (bottling, 1, 3, 6, 12 months after bottling).
* $p < 0.05$

Lagrein rosé

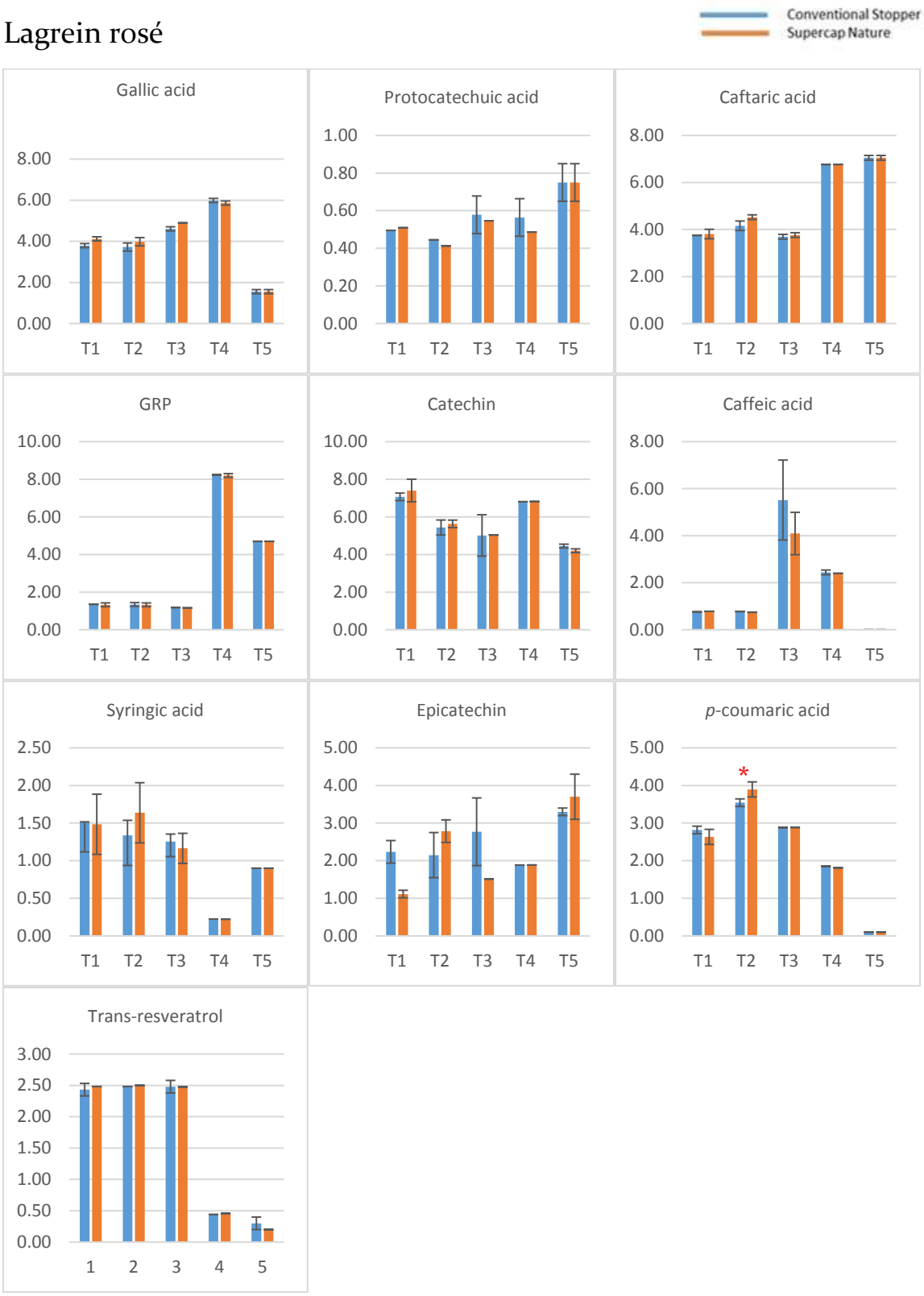


**Figure 17**. Non-anthocyanin concentrations (mg/l) in Lagrein rosé wines at the sampling times: T1, T2, T3, T4, T5 (bottling, 1, 3, 6, 12 months after bottling).
* $p < 0.05$

Merlot

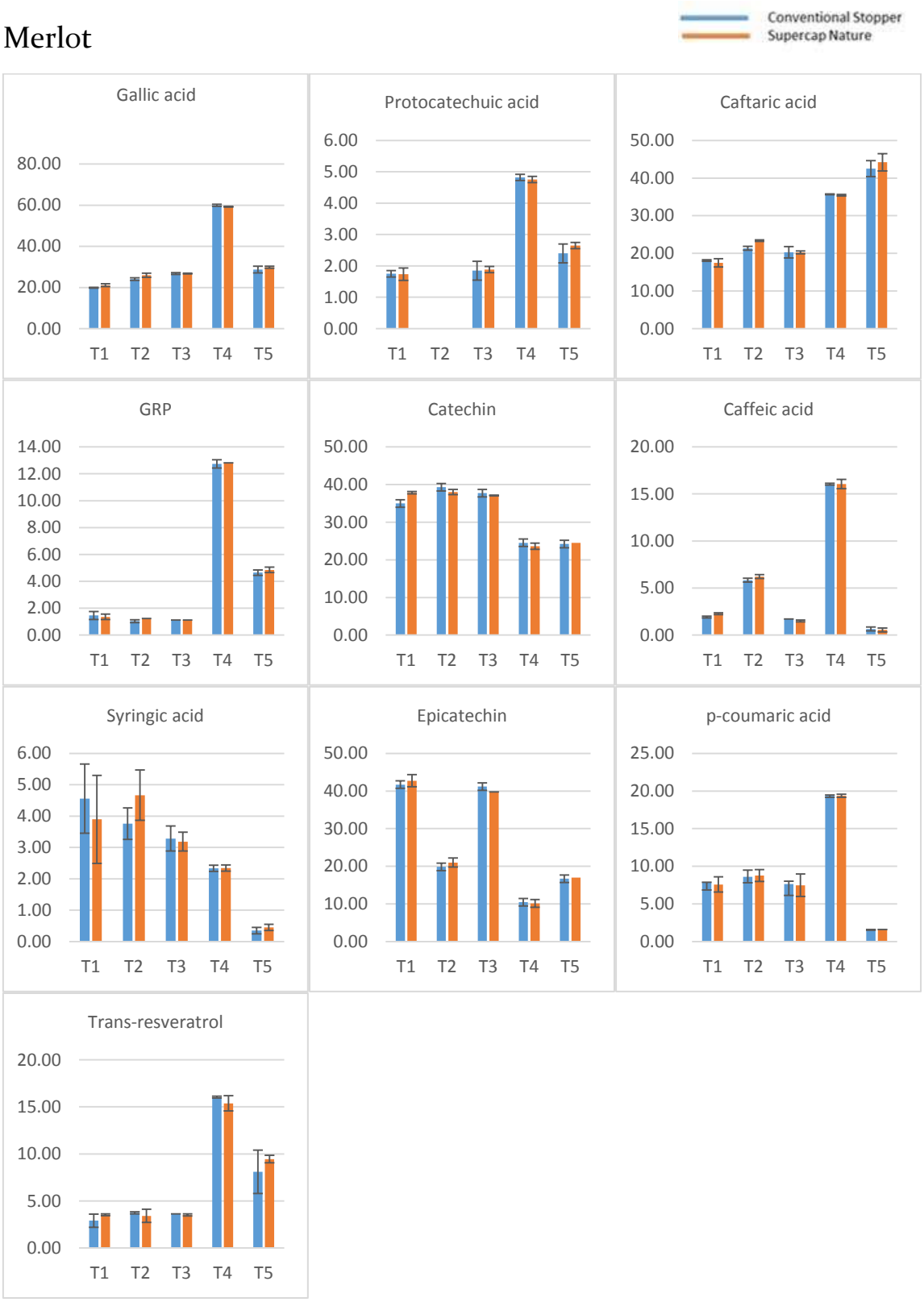


**Figure 18**. Non-anthocyanin concentrations (mg/l) in Merlot wines at the sampling times: T1, T2, T3, T4, T5 (bottling, 1, 3, 6, 12 months after bottling).
* $p < 0.05$

Santa Magdalener

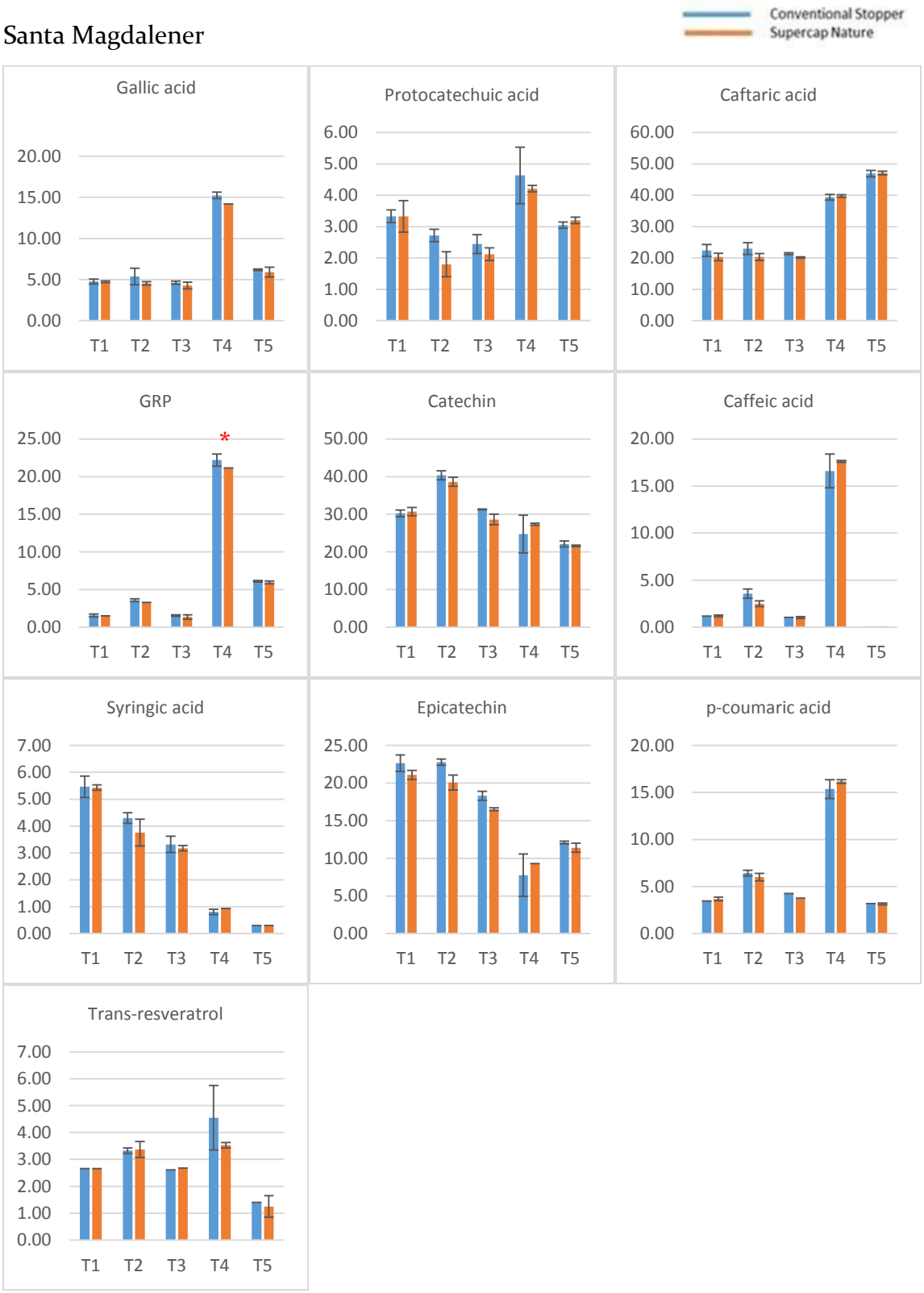


**Figure 19**. Non-anthocyanin concentrations (mg/l) in Santa Magdalener wines at the sampling times: T1, T2, T3, T4, T5 (bottling, 1, 3, 6, 12 months after bottling).
* $p < 0.05$

**Table 5**. Two-way ANOVA of the non-Anthocyanin concentrations in the four wines quantified at each sampling time (T1, T2, T3, T4, T5) (average mg/l ± st. dev.). * $p < 0.05$; ns = non significative. Conv = conventional stopper; SuperC = Supercap Nature stopper; inter = interaction; stor. period = storage period; stop. = stopper.

| | | Gallic acid | | | | | Protocatechuic acid | | | | | Caftaric acid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sampling Time | | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** |
| **Lagrein Red** | T1 | 16.2±0.5 | 16.3±0.1 | | | ns | 1.4±0 | 1.3±0.1 | | | ns | 19.9±1.2 | 18.8±0.7 | | | ns |
| | T2 | 17±0 | 18±0.5 | | | ns | 1.2±0.1 | 0.9±0 | | | ns | 20.3±0.2 | 23.7±1.2 | | | ns |
| | T3 | 18.7±1.4 | 18.4±0.1 | ns | **1932** * | ns | 1.4±0 | 2.2±0.7 | ns | **1143** * | ns | 22.1±0.1 | 20.6±0.8 | ns | **356.5** * | ns |
| | T4 | 47.6±0.7 | 46.5±0.1 | | | ns | 9.9±0.1 | 10.3±0.3 | | | ns | 41.5±3.5 | 38.8±0 | | | ns |
| | T5 | 21.7±0.2 | 21.4±0.4 | | | ns | 3.3±0.1 | 3.5±0.1 | | | ns | 47.5±0.1 | 47.2±0 | | | ns |
| **Lagrein Rosé** | T1 | 3.8±0.1 | 4.1±0.1 | | | ns | 0.5±0 | 0.5±0 | | | ns | 3.8±0 | 3.8±0.2 | | | ns |
| | T2 | 3.7±0.2 | 4±0.2 | | | ns | 0.4±0 | 0.4±0 | | | ns | 4.2±0.2 | 4.5±0.1 | | | ns |
| | T3 | 4.6±0.1 | 4.9±0 | ns | **751** * | ns | 0.6±0.1 | 0.5±0 | ns | **27** * | ns | 3.7±0.1 | 3.8±0.1 | ns | **786** * | ns |
| | T4 | 6±0.1 | 5.9±0.1 | | | ns | 0.6±0.1 | 0.5±0 | | | ns | 6.8±0 | 6.8±0 | | | ns |
| | T5 | 1.6±0.1 | 1.6±0.1 | | | ns | 0.8±0.1 | 0.8±0.1 | | | ns | 7.1±0.1 | 7.1±0.1 | | | ns |
| **Merlot** | T1 | 19.9±0.2 | 21.1±0.7 | | | ns | 1.7±0.1 | 1.7±0.2 | | | ns | 18.1±0.2 | 17.5±1.1 | | | ns |
| | T2 | 24.1±0.6 | 26±1 | | | ns | - | - | | | ns | 21.4±0.5 | 23.4±0.2 | | | ns |
| | T3 | 26.8±0.4 | 26.8±0.2 | ns | **1526** * | ns | 1.8±0.3 | 1.9±0.1 | ns | **534** * | ns | 20.3±1.5 | 20.2±0.4 | ns | **292.4** * | ns |
| | T4 | 59.9±0.5 | 59.3±0.2 | | | ns | 4.8±0.1 | 4.8±0.1 | | | ns | 35.7±0.1 | 35.4±0.2 | | | ns |
| | T5 | 28.7±1.6 | 29.8±0.5 | | | ns | 2.4±0.3 | 2.7±0.1 | | | ns | 42.5±2.1 | 44.2±2.3 | | | ns |
| **St. Magdal.** | T1 | 4.8±0.3 | 4.7±0.1 | | | ns | 3.3±0.2 | 3.3±0.5 | | | ns | 22.4±1.9 | 20.3±1.2 | | | ns |
| | T2 | 5.4±1 | 4.5±0.2 | | | ns | 2.7±0.2 | 1.8±0.4 | | | ns | 23±1.9 | 20.3±1.1 | | | ns |
| | T3 | 4.6±0.2 | 4.3±0.4 | ns | **404** * | ns | 2.4±0.3 | 2.1±0.2 | ns | **21.9** * | ns | 21.4±0.3 | 20.2±0.2 | ns | **474** * | ns |
| | T4 | 15.2±0.4 | 14.2±0 | | | ns | 4.6±0.9 | 4.2±0.1 | | | ns | 39.4±0.9 | 39.8±0.4 | | | ns |
| | T5 | 6.2±0.1 | 5.9±0.6 | | | ns | 3.1±0.1 | 3.2±0.1 | | | ns | 46.9±1 | 47.1±0.6 | | | ns |

| | | GRP | | | | | (+)-Catechin | | | | | Caffeic acid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sampling Time | | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** |
| **Lagrein Red** | T1 | 1±0.1 | 1±0.1 | | | ns | 23.7±0.1 | 24.2±2.5 | | | ns | 3.7±0.1 | 3.6±0 | | | ns |
| | T2 | 1±0.1 | 0.8±0 | | | ns | 24.9±1.2 | 24.6±2.2 | | | ns | 3.8±0 | 4.6±0 | | | ns |
| | T3 | 1±0 | 1±0.1 | ns | **626.7** * | ns | 23.9±0.3 | 18±0.4 | **14.1** * | **81.7** * | * | 4.1±0.6 | 1.7±0.1 | ns | **1132** * | * |
| | T4 | 17.3±1.8 | 16.3±0.4 | | | ns | 20±0.2 | 12.5±0.5 | | | * | 27.4±1.7 | 25.3±0.2 | | | * |
| | T5 | 1.5±0.1 | 1.5±0.1 | | | ns | 15.7±0.2 | 14.7±0.5 | | | ns | 2.1±0.1 | 2.1±0.4 | | | ns |
| **Lagrein Rosé** | T1 | 1.4±0 | 1.3±0.1 | | | ns | 7.1±0.2 | 7.4±0.6 | | | ns | 0.8±0 | 0.8±0 | | | ns |
| | T2 | 1.3±0.1 | 1.3±0.1 | | | ns | 5.4±0.4 | 5.6±0.2 | | | ns | 0.8±0 | 0.7±0 | | | ns |
| | T3 | 1.2±0 | 1.2±0 | ns | **8570** * | ns | 5±1.1 | 5±0 | ns | **29** * | ns | 5.5±1.7 | 4.1±0.9 | ns | **41** * | ns |
| | T4 | 8.2±0 | 8.2±0.1 | | | ns | 6.8±0 | 6.8±0 | | | ns | 2.4±0.1 | 2.4±0 | | | ns |
| | T5 | 4.7±0 | 4.7±0 | | | ns | 4.5±0.1 | 4.2±0.1 | | | ns | 0±0 | 0±0 | | | ns |
| **Merlot** | T1 | 1.5±0.3 | 1.4±0.2 | | | ns | 35±2 | 37.8±2.4 | | | ns | 1.9±0.1 | 2.3±0.1 | | | ns |
| | T2 | 1±0.1 | 1.2±0 | | | ns | 39.3±0.3 | 38±6.7 | | | ns | 5.8±0.2 | 6.2±0.2 | | | ns |
| | T3 | 1.1±0 | 1.1±0 | ns | **3263** * | ns | 37.7±0.7 | 37.1±5 | ns | **21.56** * | ns | 1.7±0 | 1.5±0.1 | ns | **3435** * | ns |
| | T4 | 12.7±0.3 | 12.8±0 | | | ns | 24.5±0.1 | 23.6±0.3 | | | ns | 16±0.1 | 16.1±0.5 | | | ns |
| | T5 | 4.7±0.2 | 4.9±0.2 | | | ns | 24.2±0.8 | 24.5±0.1 | | | ns | 0.7±0.2 | 0.6±0.2 | | | ns |
| **St. Magdal.** | T1 | 1.6±0.2 | 1.5±0 | | | ns | 30.2±0.9 | 30.7±1.1 | | | ns | 1.2±0 | 1.2±0.1 | | | ns |
| | T2 | 3.6±0.2 | 3.3±0 | | | ns | 40.4±1.2 | 38.6±1.2 | | | ns | 3.6±0.5 | 2.5±0.3 | | | ns |
| | T3 | 1.5±0.1 | 1.4±0.3 | ns | **2861** * | ns | 31.3±0.1 | 28.6±1.4 | ns | **56.4** * | ns | 1±0 | 1±0.1 | ns | **517** * | ns |
| | T4 | 22.2±0.8 | 21.1±0 | | | * | 24.7±5 | 27.3±0.3 | | | ns | 16.6±1.8 | 17.6±0.1 | | | ns |
| | T5 | 6.1±0.1 | 6±0.2 | | | ns | 22.1±0.8 | 21.6±0.2 | | | ns | 0±0 | 0±0 | | | ns |

| | Sampling Time | Syringic acid | | | | | (-)-Epicatechin | | | | | *p*-coumaric acid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** |
| **Lagrein Red** | T1 | 7.7±1 | 8.2±0.4 | | | ns | 26.2±1.2 | 24.7±0.5 | | | ns | 6.1±0.1 | 5.7±0.1 | | | ns |
| | T2 | 6.9±0.1 | 6.5±0.1 | | | ns | 28.2±0.2 | 25±0.3 | | | ns | 5.3±0 | 4±0.1 | | | * |
| | T3 | 6.1±0.4 | 6.3±0.5 | ns | **144** * | ns | 25.2±1.8 | 20.5±6.3 | ns | **80** * | ns | 6.1±0.2 | 6.2±0.5 | **8.2** * | **4297** * | ns |
| | T4 | 3.9±0 | 4.2±0.1 | | | ns | 5.3±0 | 4.7±0.1 | | | ns | 24.7±0.7 | 25.6±0 | | | ns |
| | T5 | 1.9±0.3 | 1.8±0 | | | ns | 10.7±0.1 | 10.2±0.4 | | | ns | 3.7±0 | 3.6±0.1 | | | ns |
| **Lagrein Rosé** | T1 | 1.5±0 | 1.5±0.4 | | | ns | 2.2±0.3 | 1.1±0.1 | | | ns | 2.8±0.1 | 2.6±0.2 | | | ns |
| | T2 | 1.3±0.2 | 1.6±0.4 | | | ns | 2.1±0.6 | 2.8±0.3 | | | ns | 3.5±0.1 | 3.9±0.2 | | | * |
| | T3 | 1.3±0.1 | 1.2±0.2 | ns | **24** * | ns | 2.8±0.9 | 1.5±0 | **5.6** * | **15** * | ns | 2.9±0 | 2.9±0 | **6.8** * | **1328** * | ns |
| | T4 | 0.2±0 | 0.2±0 | | | ns | 1.9±0 | 1.9±0 | | | ns | 1.9±0 | 1.8±0 | | | ns |
| | T5 | 0.9±0 | 0.9±0 | | | ns | 3.3±0.1 | 3.7±0.6 | | | ns | 0.1±0 | 0.1±0 | | | ns |
| **Merlot** | T1 | 4.6±1.1 | 3.9±1.4 | | | ns | 41.7±0.2 | 42.7±6.5 | | | ns | 7.9±0 | 7.6±1 | | | ns |
| | T2 | 3.8±0.5 | 4.7±0.8 | | | ns | 19.8±1.6 | 21±0.2 | | | ns | 8.6±0.9 | 8.8±0.8 | | | ns |
| | T3 | 3.3±0.4 | 3.2±0.3 | ns | **30.3** * | ns | 41.2±1.2 | 39.7±5.1 | ns | **274.1** * | ns | 7.6±0.4 | 7.5±1.5 | ns | **282.7** * | ns |
| | T4 | 2.3±0.1 | 2.3±0.1 | | | ns | 10.5±0 | 10.1±0.3 | | | ns | 19.4±0.1 | 19.4±0.2 | | | ns |
| | T5 | 0.4±0.1 | 0.5±0.1 | | | ns | 16.7±1 | 17±0.4 | | | ns | 1.5±0.1 | 1.6±0 | | | ns |
| **St. Magdal.** | T1 | 5.5±0.4 | 5.4±0.1 | | | ns | 22.7±1.1 | 21.1±0.6 | | | ns | 3.5±0 | 3.7±0.2 | | | ns |
| | T2 | 4.3±0.2 | 3.8±0.5 | | | ns | 22.8±0.4 | 20.1±1 | | | ns | 6.4±0.3 | 6±0.4 | | | ns |
| | T3 | 3.3±0.3 | 3.2±0.1 | ns | **319** * | ns | 18.3±0.6 | 16.5±0.2 | ns | **101** * | ns | 4.3±0 | 3.8±0 | ns | **705** * | ns |
| | T4 | 0.8±0.1 | 0.9±0 | | | ns | 7.8±2.8 | 9.3±0 | | | ns | 15.4±1 | 16.2±0.2 | | | ns |
| | T5 | 0.3±0 | 0.3±0 | | | ns | 12.1±0.2 | 11.4±0.6 | | | ns | 3.2±0 | 3.2±0.1 | | | ns |

| | | *Trans*-resveratrol | | | | |
|---|---|---|---|---|---|---|
| Sampling Time | | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** |
| **Lagrein Red** | T1 | 3.4±1.4 | 4±0 | | | ns |
| | T2 | 4.1±0 | 4.2±0.1 | | | ns |
| | T3 | 3.4±0.5 | 3.6±0 | ns | **160.5** * | ns |
| | T4 | 12.4±0.8 | 11.8±0.4 | | | ns |
| | T5 | 8.6±0.2 | 8.7±0.2 | | | ns |
| **Lagrein Rosé** | T1 | 2.4±0.1 | 2.5±0 | | | ns |
| | T2 | 2.5±0 | 2.5±0 | | | ns |
| | T3 | 2.4±0 | 2.5±0 | ns | **1899** * | ns |
| | T4 | 0.4±0 | 0.5±0 | | | ns |
| | T5 | 0.3±0.1 | 0.2±0 | | | ns |
| **Merlot** | T1 | 2.9±0.7 | 3.5±0.1 | | | ns |
| | T2 | 3.7±0.1 | 3.4±0.7 | | | ns |
| | T3 | 3.6±0 | 3.5±0.1 | ns | **143.3** * | ns |
| | T4 | 16±0.1 | 15.4±0.8 | | | ns |
| | T5 | 8.1±2.3 | 9.5±0.4 | | | ns |
| **St. Magdal.** | T1 | 2.7±0 | 2.7±0 | | | ns |
| | T2 | 3.3±0.1 | 3.4±0.3 | | | ns |
| | T3 | 2.6±0 | 2.7±0 | ns | **22.8** * | ns |
| | T4 | 4.5±1.2 | 3.5±0.1 | | | ns |
| | T5 | 1.4±0 | 1.3±0.4 | | | ns |

Lagrein red

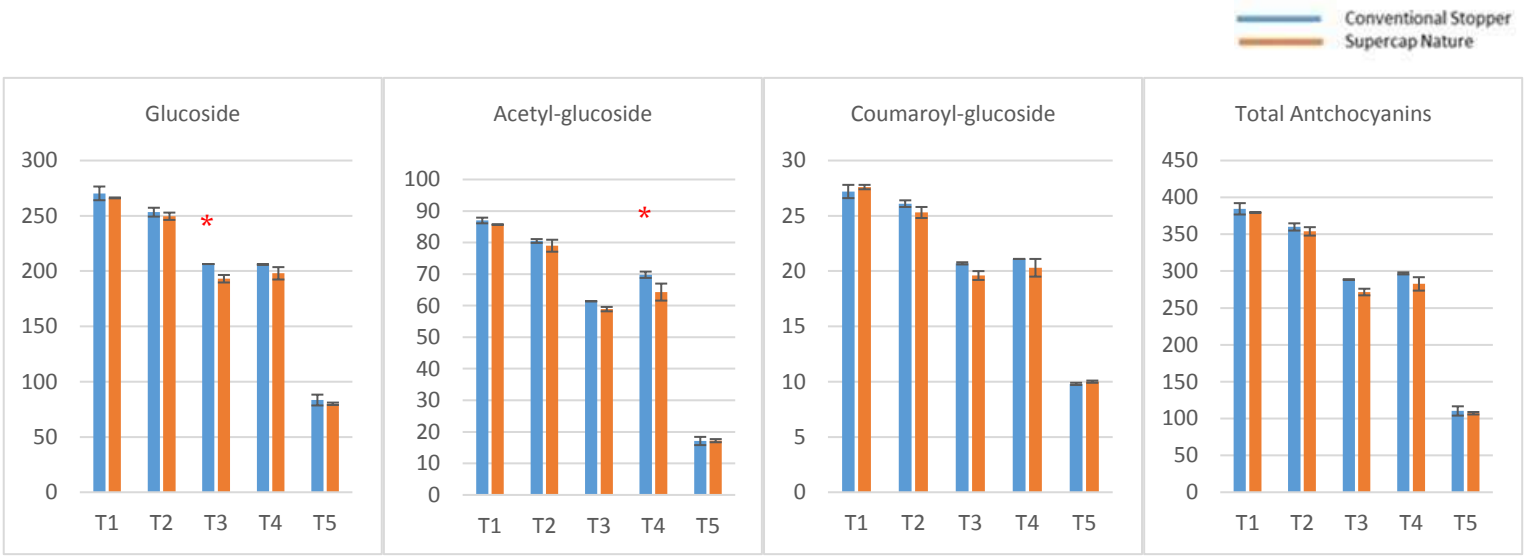


**Figure 20**. Anthocyanin concentrations (mg/l) in Lagrein red wines at: T1, T2, T3, T4, T5 (bottling, 1, 3, 6, 12 months after bottling). * $p < 0.05$

Lagrein rosé

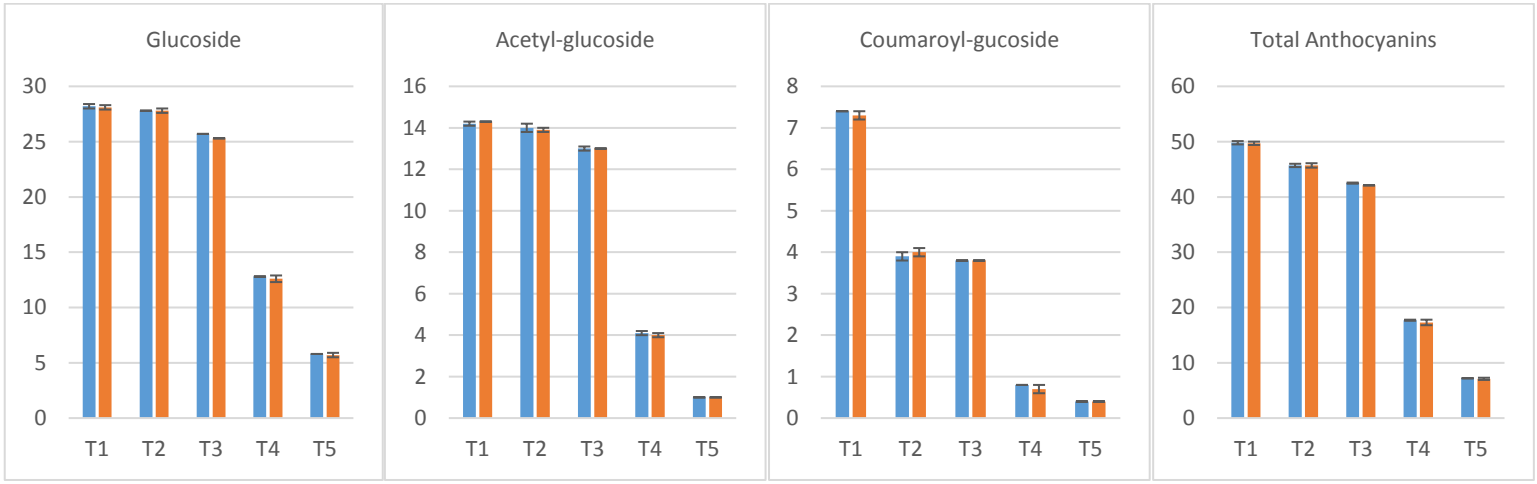


**Figure 21**. Anthocyanin concentrations (mg/l) in Lagrein rosé wines at: T1, T2, T3, T4, T5 (bottling, 1, 3, 6, 12 months after bottling). * $p < 0.05$

Merlot

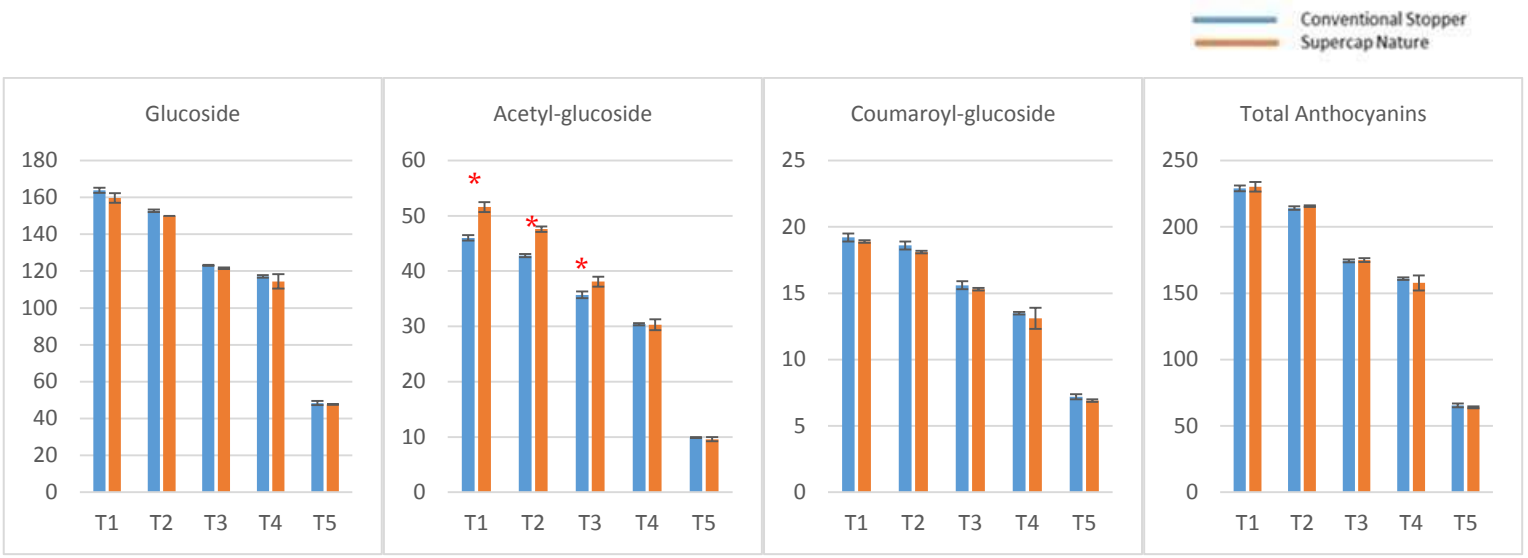


**Figure 22**. Anthocyanin concentrations (mg/l) in Merlot wines at: T1, T2, T3, T4, T5 (bottling, 1, 3, 6, 12 months after bottling). * $p < 0.05$

Santa Magdalener

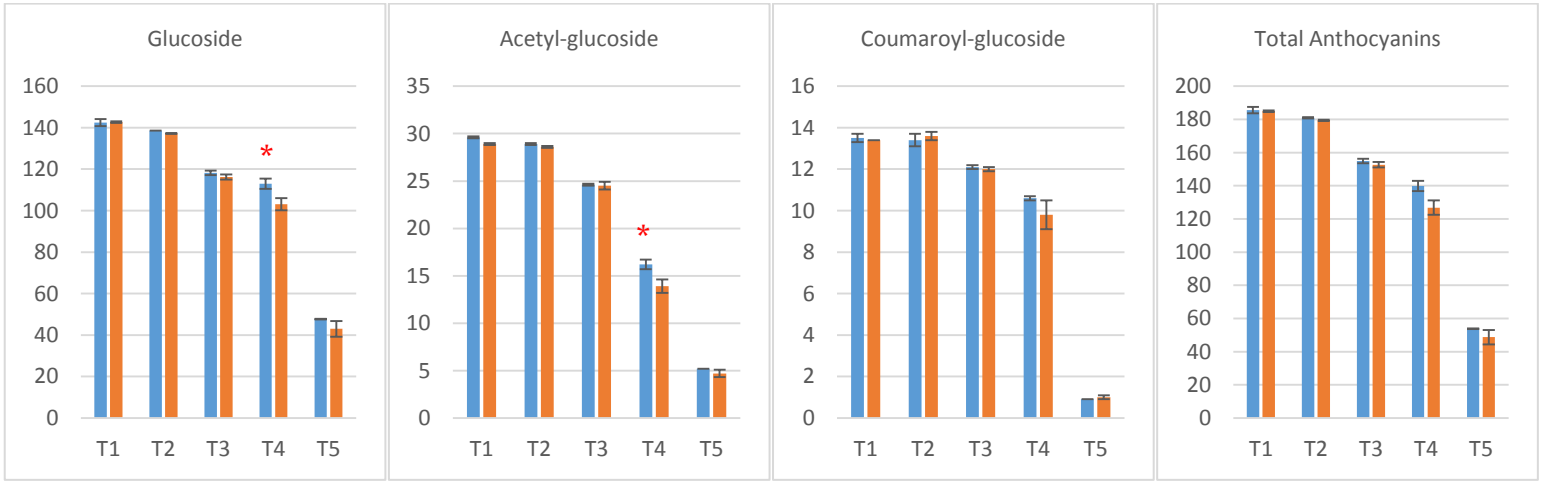


**Figure 23**. Anthocyanin concentrations (mg/l) in Santa Magdalener wines at: T1, T2, T3, T4, T5 (bottling, 1, 3, 6, 12 months after bottling). * $p < 0.05$

**Table 6**. Two-way ANOVA of the anthocyanin concentrations in the four wines quantified at each sampling time (T1, T2, T3, T4, T5) (average mg/l ± st. dev.).

* $p < 0.05$; ns = non significative; Conv = conventional stopper; SuperC = Supercap Nature stopper; inter = interaction; stor. period = storage period; stop. = stopper.

| | | *Glucoside* | | | | | *Acetyl-glucoside* | | | | | *Cumaroyl-glucoside* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sampling Time | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** | Conv. | SuperC. | ***F* (inter.)** | ***F* (stor. period)** | ***F* (stopp.)** |
| **Lagrein Red** | T1 | 270.3±6.2 | 266.2±0.4 | | | ns | 87±0.9 | 85.7±0.1 | | | ns | 27.2±0.6 | 27.6±0.2 | | | ns |
| | T2 | 253.3±4 | 249.6±3.3 | | | ns | 80.5±0.6 | 79±1.9 | | | ns | 26.1±0.3 | 25.3±0.5 | | | ns |
| | T3 | 206.4±0 | 193.1±3.4 | ns | **1481** * | * | 61.4±0 | 58.9±0.7 | ns | **1694** * | ns | 20.7±0.1 | 19.6±0.4 | ns | **1130** * | ns |
| | T4 | 206±0.4 | 198±5.6 | | | ns | 69.8±1 | 64.3±2.7 | | | * | 21.1±0 | 20.3±0.8 | | | ns |
| | T5 | 83.3±5 | 80±1.2 | | | ns | 17.1±1.3 | 17.2±0.4 | | | ns | 9.8±0.2 | 10±0.3 | | | ns |
| **Lagrein Rosé** | T1 | 28.2±0.2 | 28.1±0.2 | | | ns | 14.2±0.1 | 14.3±0 | | | ns | 7.4±0 | 7.3±0.1 | | | ns |
| | T2 | 27.8±0 | 27.8±0.2 | | | ns | 14±0.2 | 13.9±0.1 | | | ns | 3.9±0.1 | 4±0.1 | | | ns |
| | T3 | 25.7±0 | 25.3±0 | ns | **34170*** | ns | 13±0.1 | 13±0 | ns | **20423** * | ns | 3.8±0 | 3.8±0 | ns | **10927** * | ns |
| | T4 | 12.8±0 | 12.6±0.3 | | | ns | 4.1±0.1 | 4±0.1 | | | ns | 0.8±0 | 0.7±0.1 | | | ns |
| | T5 | 5.8±0 | 5.7±0 | | | ns | 1±0 | 1±0 | | | ns | 0.4±0 | 0.4±0 | | | ns |
| **Merlot** | T1 | 163.8±1.4 | 159.7±2.6 | | | ns | 46±0.5 | 51.6±0.9 | | | * | 19.2±0.3 | 18.9±0.1 | | | ns |
| | T2 | 152.7±0.7 | 149.9±0 | | | ns | 42.8±0.3 | 47.6±0.5 | | | * | 18.6±0.3 | 18.1±0.1 | | | ns |
| | T3 | 123.1±0.2 | 121.5±0.4 | ns | **3439** * | ns | 35.7±0.6 | 38.1±0.9 | **15.5** * | **1941** * | * | 15.6±0.3 | 15.3±0.1 | ns | **979** * | ns |
| | T4 | 117.1±0.7 | 114.4±3.9 | | | ns | 30.4±0.2 | 30.3±1 | | | ns | 13.5±0.1 | 13.1±0.8 | | | ns |
| | T5 | 48.4±1.1 | 47.6±0.4 | | | ns | 9.9±0.1 | 9.6±0.4 | | | ns | 7.2±0.1 | 6.8±0 | | | ns |
| **St. Magd.** | T1 | 142.5±1.7 | 142.7±0.4 | | | ns | 29.6±0.1 | 28.9±0.1 | | | ns | 13.5±0.2 | 13.4±0 | | | ns |
| | T2 | 138.6±0 | 137.2±0.1 | | | ns | 28.9±0.1 | 28.6±0.1 | | | ns | 13.4±0.3 | 13.6±0.2 | | | ns |
| | T3 | 118.2±1.1 | 116.2±1.2 | **4.5** * | **4767** * | ns | 24.6±0.1 | 24.5±0.4 | **5.6** * | **2971** * | ns | 12.1±0.1 | 12±0.1 | ns | **1867** * | ns |
| | T4 | 113±2.5 | 103.1±2.9 | | | * | 16.2±0.5 | 13.9±0.7 | | | * | 10.6±0.1 | 9.8±0.7 | | | ns |
| | T5 | 47.6±0.3 | 43.1±3.9 | | | ns | 5.2±0 | 4.6±0.4 | | | ns | 0.9±0 | 1±0.1 | | | ns |

### *2.3.3 Evolution of volatile compounds during the storage in bottle*

In the three red and in the rosé wine a total of twenty-six volatile compounds were identified (Table 7) and their abundance was expressed as relative percentage of the internal area (Supporting Information Tables). The most represented class was the esters with seventeen compounds, followed by alcohols, acids and terpens.

**Table 7**. Identified volatile compounds through SPME/GC-MS.

| Elution order | Esters | Linear Retention indices |
|---|---|---|
| 2 | Ethyl butanoate | 803[l] |
| 3 | Butanoic acid, 2-methyl ethyl este | 846[l] |
| 4 | Butanoic acid, 3-methyl ethyl este | 859[b] |
| 6 | Isopentyl acetate | 876[l] |
| 7 | 4-Ethylbenzoic acid, 2-butylester | - |
| 10 | Ethyl hexanoate | 999[l] |
| 11 | Hexyl acetate | 1011[l] |
| 14 | 4-Methyl benzaldehyde | 1076[d] |
| 16 | 4-Ethylbenzaldehyde | 1163[e] |
| 18 | Diethyl succinate | 1179[f] |
| 20 | Methyl salicylate | 1192[l] |
| 21 | Ehtyl octanoate | 1194[h] |
| 22 | Benzenacetic acid ethyl ester | 1243[l] |
| 23 | 2-Phenylethylacetate | 1255[l] |
| 24 | Ethyl decanoate | 1392[l] |
| 25 | Ethyl dodecanoate | 1554[l] |
| 26 | Ethyl hexadecanoate | 1992[i] |

| Elution order | Alcohols | Linear Retention indices |
|---|---|---|
| 5 | 1-Hexanol | 865[l] |
| 8 | 1-Heptanol | 969[l] |
| 9 | 1-Octen-3ol | 980[a] |
| 13 | 2-Ethyl hexanol | 1028[c] |
| 15 | Octanol | 1070[l] |
| 17 | 2-Phenyl ethyl alcohol | 1112[a] |
| | **Acids** | |
| 1 | Acetic acid | 599[a] |
| 19 | Octanoic acid | 1180[g] |
| | **Terpens** | |
| 12 | Limonene | 1020[l] |

[a] Klesk and Qian, 2003; [b] Kim T.H., Kim T.H., et al., 2002; [c] Sampaio and Nogueira, 2006; [d] Xu, van Stee, et al., 2003; [e] Schirack, Drake, et al., 2006 ; [f] Su, Ho, et al., 2006; [g] Passos X.S., Castro A.C.M., et al., 2003; [h] Flamini, Luigi Cioni, et al., 2003; [i] Boulanger, Chassagne, et al., 1999; [l] Beaulieu and Grimm, 2001.

During the bottle storage more than 80% of the total abundance was represented by seven compounds: isopentyl acetate, 1-hexanol, ethyl hexanoate, 2-phenylethyl alcohol, diethyl succinate, ethyl octanoate and ethyl decanoate. Over twelve months, the modification of the volatile composition was primarily due to the evolution of these compounds. Ethyl octanoate and ethyl decanoate showed a decreasing trend in the early period of bottle storage (T1-T4) followed by an increase until the twelfth month (T5) in the three red wines (Figure 24.).

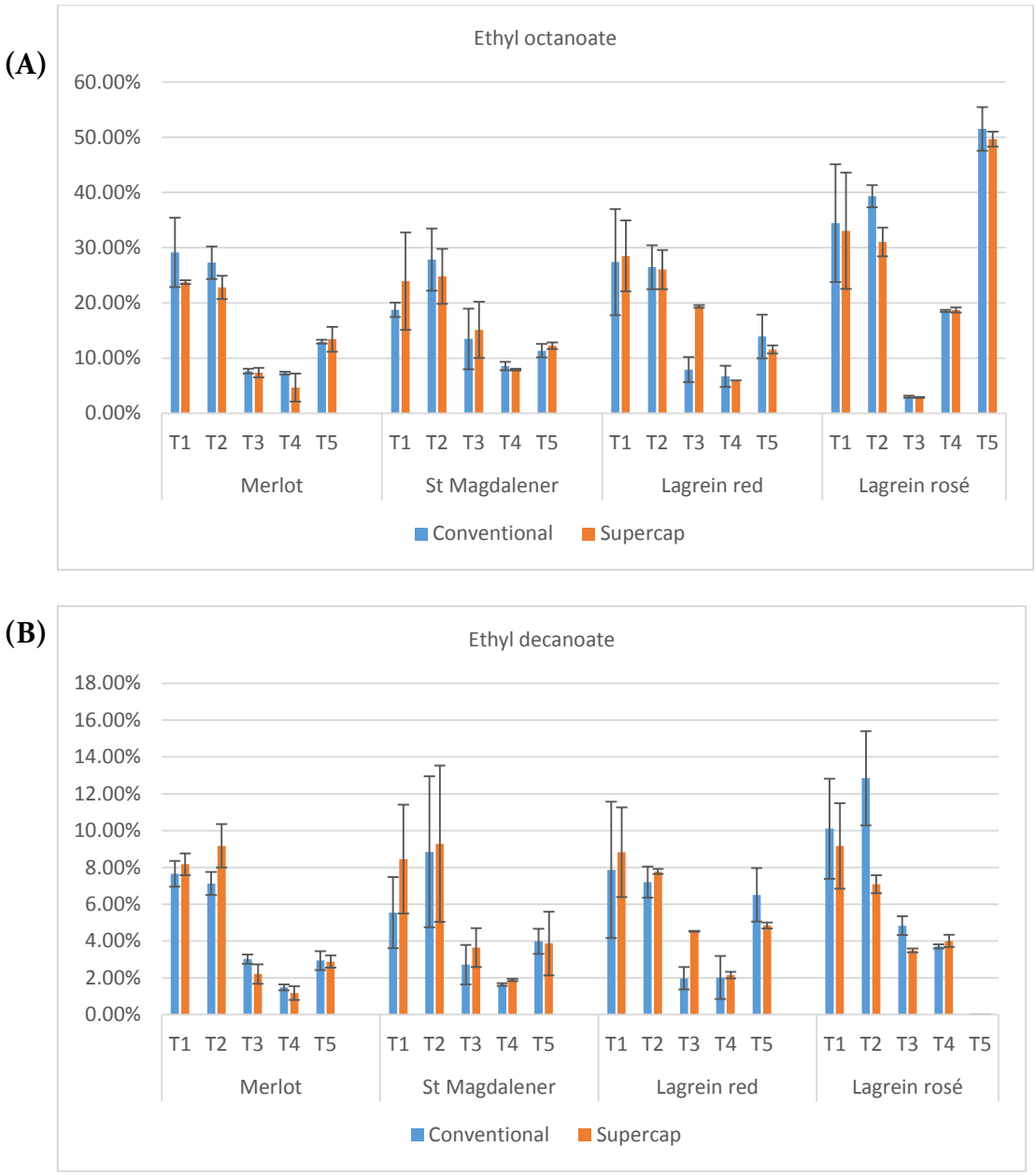


**Figure 24.** Abundances of the **(A)** Ethyl octanoate and **(B)** Ethyl decanoate during the five sampling times: T1, T2, T3, T4, T5.

The opposite evolution pattern was detected for 1-hexanol and diethyl succinate (Figure 25.).

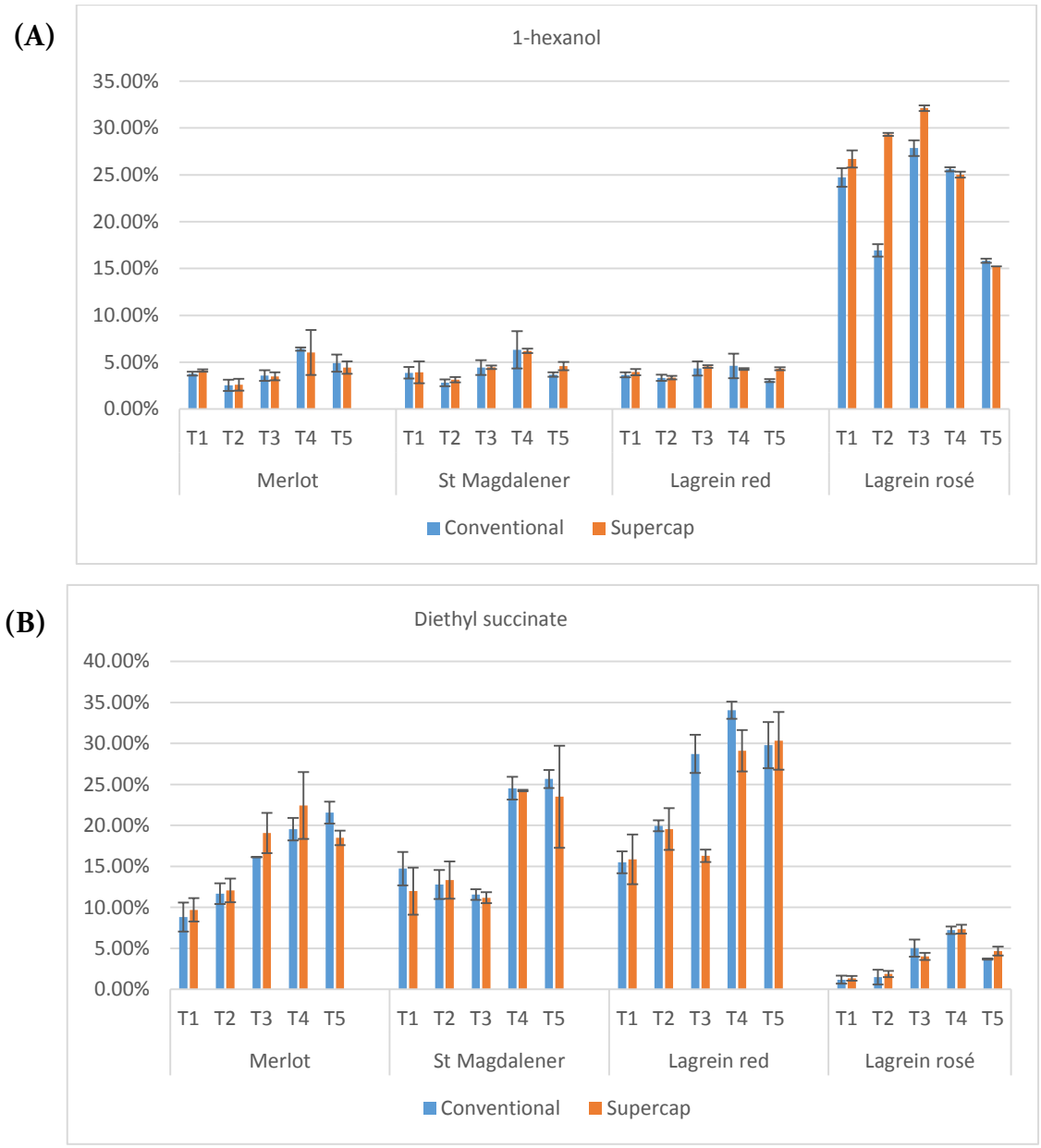


**Figure 25.** Abundances of the **(A)** 1-hexanol and **(B)** diethyl succinate during the five sampling times: T1, T2, T3, T4, T5.

Isopentyl acetate increased after one month (T2) of bottle storage in the three red wines and reached its maximum of abundance at three month (T3) (Figure 26.), probably as a consequence of acid-catalyzed reactions involving fatty acid esters and resulting in the production of acetate esters (Moio *et al.*, 2004; Ugliano *et al.*, 2008).

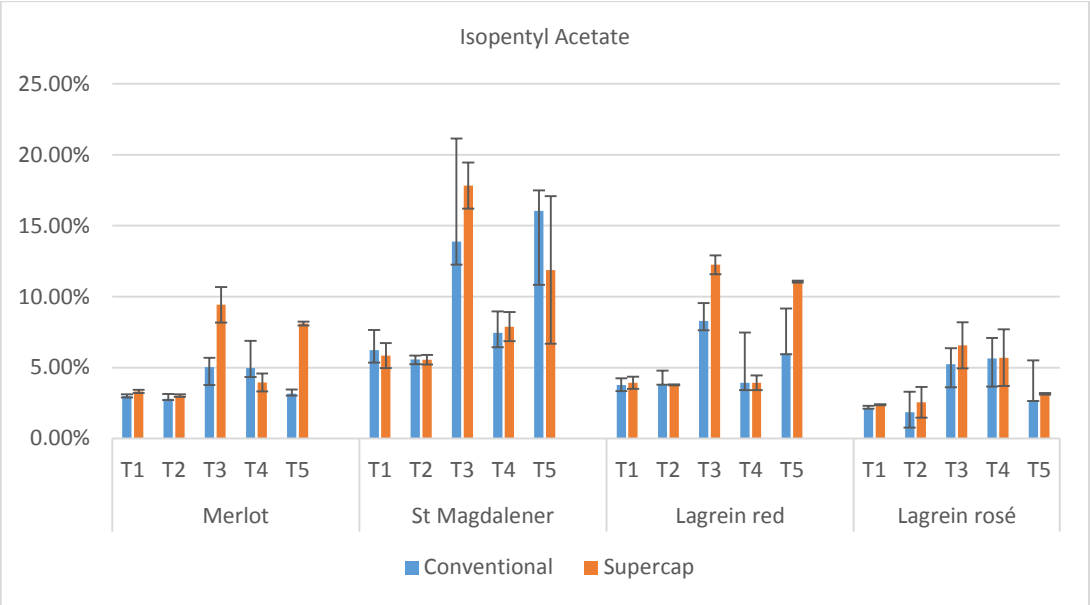


**Figure 26.** Abundances of the isopentyl acetate during the five sampling times: T1, T2, T3, T4, T5.

The total abundance of the alcohols decreased in all the four wines during the twelve months of storage in bottle (Figure 27.). This reduction was also reported in previous studies (Escudero *et al.*, 2000; Culleré *et al.*, 2007; Fedrizzi *et al.*, 2011) and can be explained as the result of the oxidation of aliphatic alcohols to aldehydes and afterward to acids.

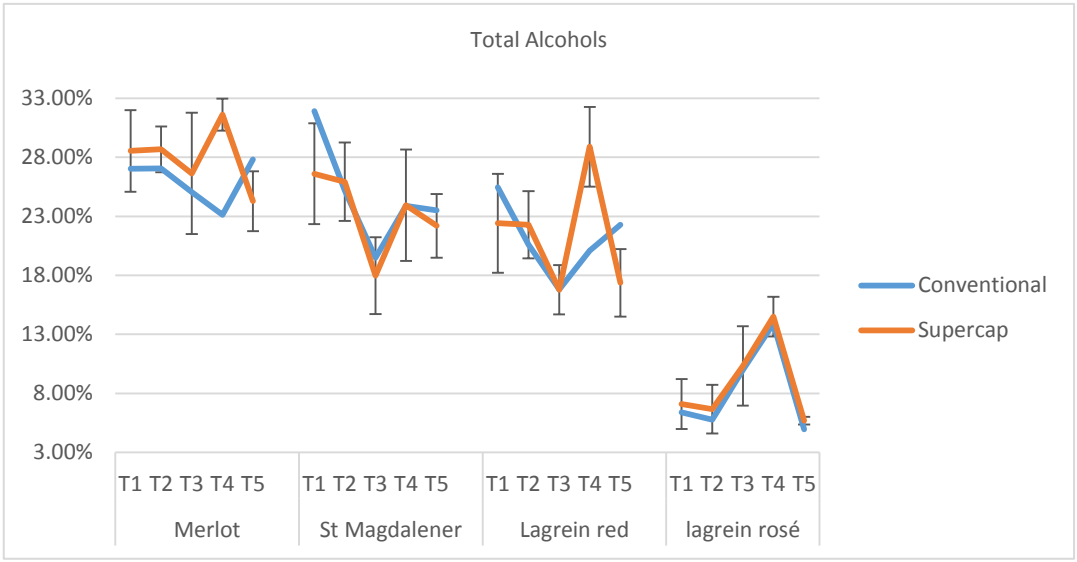


**Figure 27.** Total abundances of alcohols at the five sampling times: T1, T2, T3, T4, T5.

Concerning the total amount of esters, Merlot distinguished for a slight reduction during the twelve months in wine closed with the conventional stopper. Whereas, the supercap stopper showed a higher abundance at the end of the storage compared to

the beginning in all the four wines (Figure 28.). This latter trend, even if less common than the one detected in Merlot closed with conventional stopper, can occur as the result of the esterification of branched acids to form ethyl esters (Ferreira *et al.*, 1997; Hernanz *et al.*, 2009; Makhotkina *et al.*, 2012).


**Figure 28.** Total abundances of esters at the five sampling times: T1, T2, T3, T4, T5.

### *2.3.4 Hierarchical Clustering analysis (HCA)*

The HCA was performed to assess the overall similarities and differences between the wines using the phenolic and volatile compounds identified as variables. The dendrogram clearly segregated the Lagrein rosé wine in a single branch of the diagram, separated from the three red wines (Figure 29). Merlot, Lagrein red and Santa Magdalener wines were clustered at close hierarchical distance at T1 and T2, highlighting similarities to each other. Samples obtained after 3 and 12 months after bottling (T3 and T5) were segregated in two adjacent branches whereas sample after 6 months from bottling were isolated in an individual branch. Evidently, considering the 12 months of bottle storage the evolution of the wines started after 3 months (T3) and reached their maximum differentiation after 6 months (T4). At 12 months of bottle storage, the composition of the wines changed again and returned to be more similar to the initial one. Regarding the type of stopper, the dendrogram always grouped the wines closed with the two comparative stoppers at close hierarchical distance, explaining the similar influence on the phenolic and volatile composition.

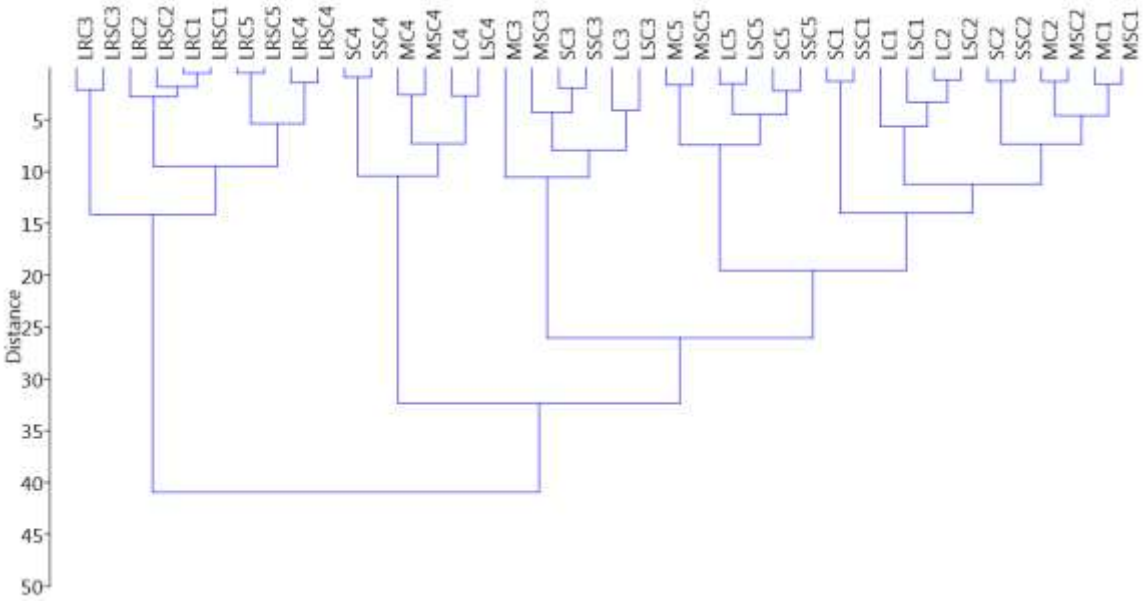


**Figure 29**. Hierarchical Cluster Analysis of the four wines during 12 months of bottle storage. SAMPLES MC=Merlot Conventional stopper; MSC= Merlot Supercap stopper; LC= Lagrein Conventional; LSC= Lagrein Supercap; SC= St. Magdalener; SSC= St. Magdalener Supecap; LRC= Lagrein rosé Conventional; LRSC= Lagrein rosé Supercap. Sampling time: (1) = bottling time; (2) = 1 month after bottling; (3) = 3 months after bottling; (4) = 6 months after Sampling time (5) = 12 months after bottling.

### *2.3.5 Principal component analysis (PCA)*

The PCA was performed in order to confirm the results obtained from the HCA and to assess further relations between the phenolic and volatile compounds and type of stopper during the bottle storage. All the identified phenolic and volatiles were used as variables. The PCA plot showed the loading and the scores in the bi-dimensional space. The first two principal components explained together 47% of the model (PC-1 30%, PC-2 17%) thus also PC-3 (15%) was considered for a total variance of 63%. PC-1 vs PC-2 bi-plot (Figure 30), grouped rosé samples in the lower-left part of the plot. Whereas, the red wine samples were grouped according with the storage period. Samples stored for six months (T4) were clearly isolated in the lower-right quadrant, whereas the wines sampled at bottling (T1) and after one month (T2) were grouped together, similarly to those wines analyzed at three and twelve months (T3 and T5). PC-1 differentiated rosé wines from the red samples whereas, PC-2 and PC-3 separated the three red wines in base of the storage period. Considering the loadings, the anthocyanin compounds characterized the positive axis of PC-2 showing a correlation

with T1 and T2 samples. Some of the volatile compounds related to fresh fruity and floral notes such as, limonene (12), ethyl octanoate (21) and ethyl decanoate (24) positively correlated with samples stored for short period (T1 and T2).

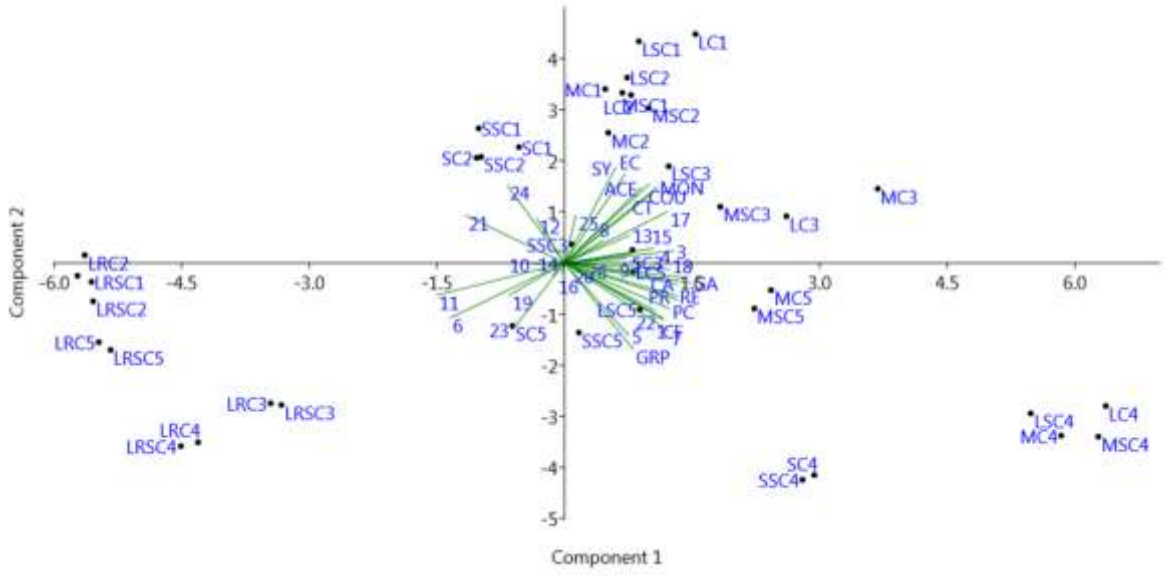


**Figure 30**. Principal Component Analysis of the three red and the rosé wine during 12 months of bottle storage (PC-1 versus PC-2).

Phenolic compounds: MON = Glucoside Anthocyanins; ACE = Acetyl-glucoside Anthocyanins; COU = coumaroyl-glucoside anthocyanins; GA = gallic acid; PR = protocatechuic acid; GRP = glutathionyl caftaric acid; CA = caftaric acid; CF = caffeic acid; CT = catechin; EC = epicatechin; SY = syringic acid; PC = p-coumaric acid; RE = resveratrol.

Volatile compounds are named with a number from 1 to 26 as listed in Table 7

On the other hand, some volatile compounds connected with oxidation reaction such as acetic acid (1) and with developed aroma named phenylaceticacid ethyl ester (23) and diethyl succinate (18), distinguished samples stored for longer period (T4 and T5) in both the bi-plot (PC1 vs PC-2 and PC-1 vs PC-3 (Figure 31).

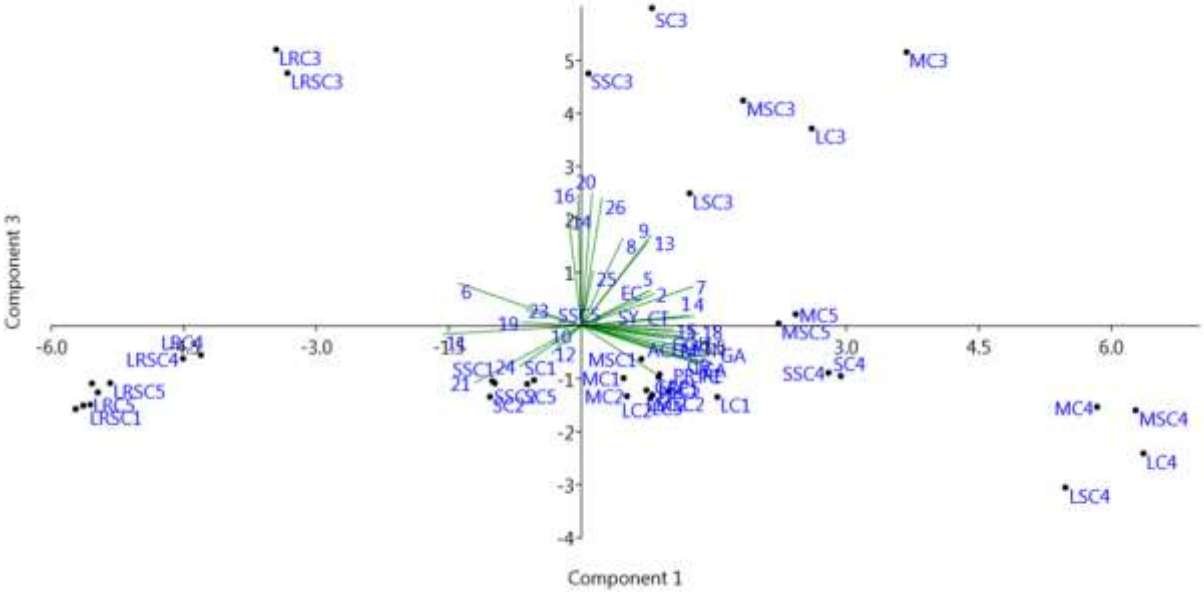


**Figure 31.** Principal Component Analysis of the three red and the rosé wine during 12 months of bottle storage (PC-1 versus PC-3).

Phenolic compounds: MON = Glucoside Anthocyanins; ACE = Acetyl-glucoside Anthocyanins; COU = coumaroyl-glucoside anthocyanins; GA = gallic acid; PR = protocatechuic acid; GRP =glutathionyl caftaric acid; CA = caftaric acid; CF = caffeic acid; CT = catechin; EC = epicatechin; SY = syringic acid; PC = p-coumaric acid; RE = resveratrol.

Volatile compounds are named with a number from 1 to 26 as listed in Table 7

The PCA successfully scattered the samples depending on the storage period, whereas the sample obtained with the two compared stoppers were never clearly separated by a principal component. This explain the similar influence of the different stoppers on the phenolic and volatile composition at each step of bottle storage. These results confirmed the clustering observed from the HCA with the samples segregated in accordance with the storage time. Consistently, the type of stoppers did not differentiate the phenolic and volatile concentration during the bottle storage period, also in agreement with the similar ORT values for the two stoppers previously described.

### *2.3.6 Sensory analysis*

The discriminant triangle test analysis was performed to assess the perception of possible differences between the wines closed with the two types of stoppers at each

sampling time (Table 8). The sensitivity parameters of the test were set at α = 0.5, β = 0.20 and $p_d$ = 50%. Eighteen assessors attended the tasting sessions and they were asked to choose the odd sample between three glasses for each of the four wine under investigation. The results of the triangle test showed differences for St. Magdalener wine at the bottling time (T1) only in one of the two tasting sessions with twelve correct answers out of eighteen assessors. A significative number of assessors also recognized Merlot wine samples in both tasting sessions with 10 and 12 correct answers, respectively. At the tasting sessions of the samples stored for one month (T2) and 3 months (T3) did not reach the required number of correct answers to differentiate the two samples closed with the different stoppers for any of the four wines.

**Table 8.** Triangle test for the four wines during the five sampling sessions.

| | | Lagrein rosè | St Magdalener | Lagrein red | Merlot |
|---|---|---|---|---|---|
| T1 | **Correct Answers (Session I)** | 7 | 7 | 4 | 10 |
| | | ns | ns | ns | * |
| | **Correct Answers (Session II)** | 9 | 12 | 5 | 12 |
| | | ns | * | ns | * |
| T2 | **Correct Answers (Session I)** | 3 | 6 | 5 | 8 |
| | | ns | ns | ns | ns |
| | **Correct Answers (Session II)** | 2 | 7 | 4 | 4 |
| | | ns | ns | ns | ns |
| T3 | **Correct Answers (Session I)** | 6 | 7 | 3 | 5 |
| | | ns | ns | ns | ns |
| | **Correct Answers (Session II)** | 1 | 2 | 4 | 3 |
| | | ns | ns | ns | ns |
| T4 | **Correct Answers (Session I)** | 7 | 8 | 4 | 12 |
| | | ns | ns | ns | * |
| | **Correct Answers (Session II)** | 4 | 6 | 4 | 11 |
| | | ns | ns | ns | * |
| T5 | **Correct Answers (Session I)** | 4 | 1 | 3 | 0 |
| | | ns | ns | ns | ns |
| | **Correct Answers (Session II)** | 1 | 2 | 2 | 1 |
| | | ns | ns | ns | ns |

* Needed correct answers: 10; needed assessors: 18; $\alpha = 0.05$; $\beta = 0.20$.

After six months of bottle storage (T4) the triangle test resulted successful in the identification of the odd Merlot sample in both tasting sessions with eleven and twelve correct answers respectively, whereas the assessors did not recognize the odd sample for the other three wines. The sufficient correct answers were not reached at

the tasting session after twelve months of storage (T5). An overall view of the tasting sessions highlighted few cases of significative differences. Considering the tasting sessions of the wines just bottled (T1) and closed with the two different stoppers it is not possible to point the influence on the differences perceived to the type of stopper. Whereas, further investigations were performed to identify the compounds possibly responsible of the correct discrimination of the Merlot wine after 6 months of bottle storage. In this regard, a preliminary PCA was performed to assess the influence of phenols and volatiles individually. It resulted that phenols were less effective in separating the different types of stopper at each sampling time comparing to the volatiles. Thus, the investigation focused on the volatile profile of the three red wines (rosé was excluded for its low separation between the types of stopper). Eleven compounds (Acetic acid; 1-Hexanol; Isopentyl acetate; Ethyl hexanoate; Hexyl acetate; Octanol; 2-Phenyl ethyl alcohol; Diethyl succinate; Ehtyl octanoate; Ethyl decanoate; Ethyl dodecanoate) were selected according to the availability of the odor thresholds reported in literature to better evaluate their impact on the aromatic perception. The abundances of the selected volatiles were divided by their threshold value. Data were normalized and the PCA was performed for each of the three wines individually. The aim was of identifying the molecules discriminating the odd Merlot wine that was recognized by the assessors after 6 months of bottle storage (T4). The results of the three PCA showed that PC1-PC3 better separated the two types of stoppers. The bi-plot for the Merlot wines (Figure 32) showed that at 6 months of storage the sample closed with the Supercap stopper (4MSC)was characterized by the isopentyl acetate (6), diethyl succinate (18) and ethyl dodecanoate (25) whereas, sample closed with the conventional stopper (4MC) was characterized by acetic acid (1), 1-hexanol (5) and octanol (15). The comparison with the bi-plot obtained from the Lagrein red and the Santa Magdalener samples (Figure 33; 34) highlighted that the ethyl dodecanoate was reported only for the Merlot supercap sample and it did not show at T4 in the other two wines. Even if the abundance detected for this compound was low (considering the total abundance of the volatile profile; S.I. tables) the result of the PCA allowed speculating about the influence of the ethyl dodecanoate on the correct discrimination of the odd Merlot sample closed with the Supercap Nature stopper. Hypothetically, this volatile compound and its interaction with other volatile

molecules affected the aromatic profile of the Merlot wine after six months of storage in bottle.

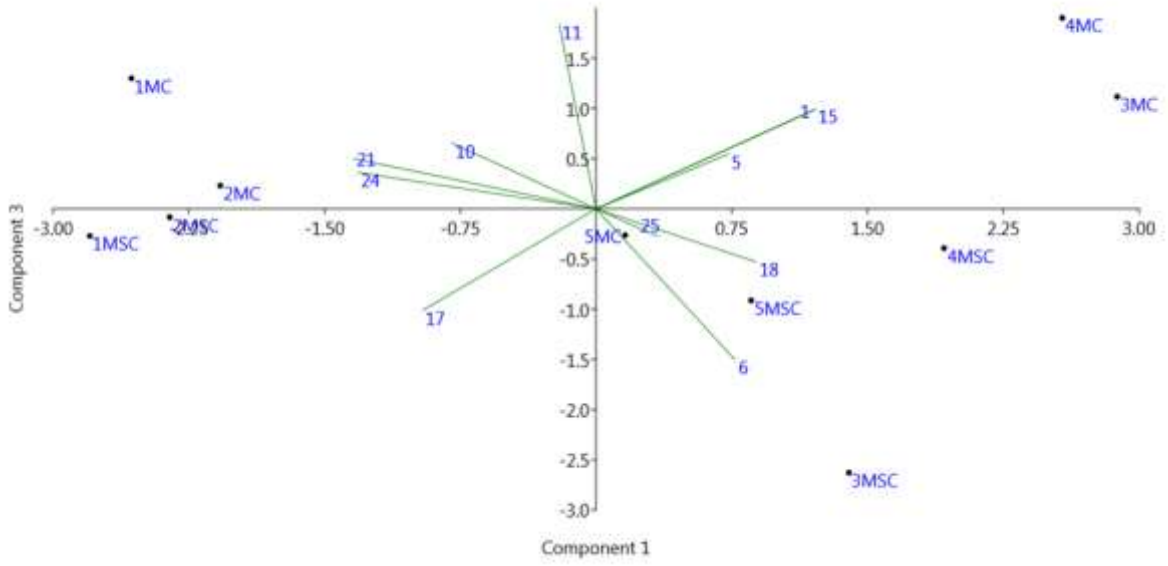


**Figure 32.** Bi-plot of the Merlot wine samples. Variables are named in accordance with Table 7. Samples: MC = Merlot conventional stopper. MSC = Merlot Supercap stopper. 1; 2; 3; 4; 5 = Bottling time, 1, 3, 6, 12 months after bottling. PC-1 (46%); PC-3 (14%).

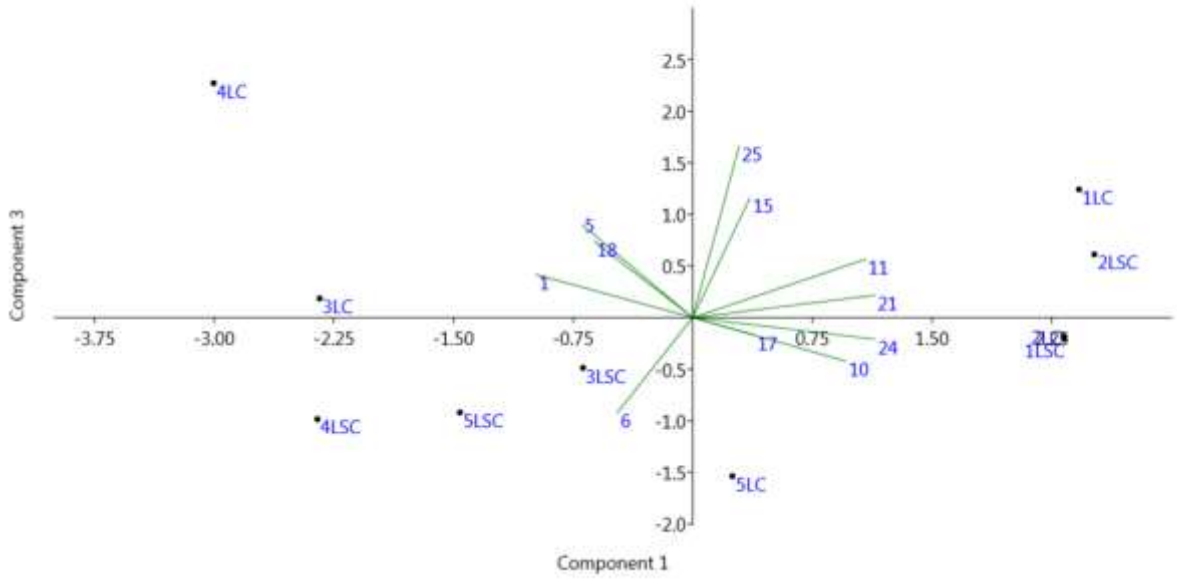


**Figure 33.** Bi-plot of the Lagrein red wine samples. Variables are named in accordance with Table 7. Samples: LC = Lagrein conventional stopper. LSC = Lagrein Supercap stopper. 1; 2; 3; 4; 5 = Bottling time, 1, 3, 6, 12 months after bottling. PC-1 (46%); PC-3 (18%).

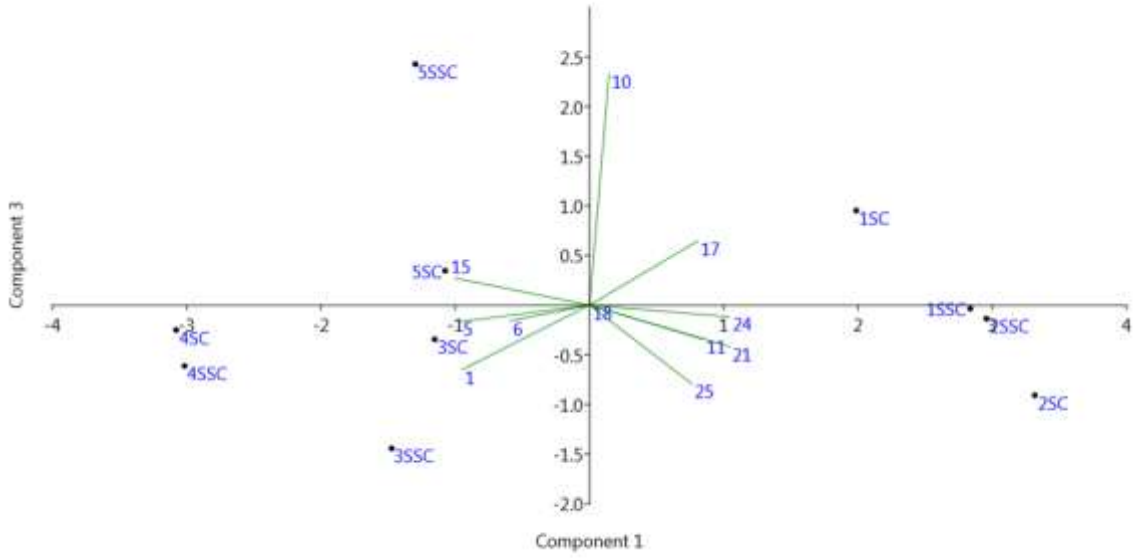


**Figure 34.** Bi-plot of the Santa Magdalener wine samples. Variables are named in accordance with the Table 7. Samples: SC = Santa Magdalener conventional stopper. SSC = Santa Magdalener Supercap stopper. 1; 2; 3; 4; 5 = Bottling time, 1, 3, 6, 12 months after bottling. PC-1 (57%); PC-3(10%).

## 2.4 Conclusion

This work explored the influence of the type of stopper and the storage period on the phenolic and volatile profile during 12 months of bottle storage. Further knowledge on the evolution of the phenolic and volatile compounds in three red and one rosé wine from Northern Italy were provided.

Non-anthocyanin phenolic compounds named gallic acid, caffeic acid, *p*-coumaric acid, *trans*-resveratrol, GRP and protocatechuic acid resulted to have a common evolution trend in the four wines. During the first three months of bottle storage (T1, T2, T3) the concentrations remain constant. At six months (T4) a net increase followed by a clear reduction at 12 months (T5) was reported. The total anthocyanin content clearly decreased during the bottle storage. The abundance of the cyclic proanthocyanidins was higher in Lagrein red and Santa Magdalener wine. However, in the four wines, significant differences were not detected between the two types of stoppers.

The volatile profile was characterized by the high concentrations of isopentyl acetate, 1-hexanol, ethyl hexanoate, 2-phenylethyl alcohol, diethyl succinate, ethyl octanoate and ethyl decanoate. During the bottle storage the modification of the volatile

composition was primary due to the evolution of these compounds. The total abundance of the alcohols decreased in all the four wines. The esters showed a reduction in Merlot wine in comparison to Lagrein rosé, Lagrein red and Santa Magdalener wines which showed an increasing concentration during the storage period. The triangle test resulted significative only for Santa Magdalener and Merlot wines at bottling time and after six months of bottle storage. The further investigation at the sampling time T4 through the PCA showed that presumably the ethyl dodecanoate (25) and its interaction with other volatile compounds allowed the discrimination of the Merlot wine closed with the Supercap Nature stopper from the conventional one.

However, in most of the cases at each sampling time the two types of stopper showed a similar influence on the wines. Notably, the evolution of the phenolic and volatile concentrations resulted foremost influenced by the non-oxygen mediated reactions occurring during the bottle storage period.

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# Supporting Information

**Table SI 1a**. Relative abundances of the volatile compounds at the five sampling times (T1, T2, T3, T4, T5) in Merlot wines.

| | | T1 | | T2 | | T3 | | T4 | | T5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Merlot | Conv | SuperC | Conv | SuperC | Conv | SuperC | Conv | SuperC | Conv | SuperC |
| 1 | Acetic acid | 0.00% | 0.00% | 0.00% | 0.00% | 5.16% | 0.88% | 3.80% | 3.20% | 0.47% | 0.75% |
| 2 | Ethyl butanoate | 2.33% | 2.30% | 1.91% | 2.13% | 3.24% | 1.13% | 2.24% | 2.38% | 2.61% | 1.83% |
| 3 | Butanoic acid, 2-methyl ethyl ester | 0.31% | 0.31% | 0.39% | 0.51% | 0.33% | 0.22% | 0.61% | 0.52% | 0.58% | 0.64% |
| 4 | Butanoic acid, 3-methyl ethyl ester | 0.48% | 0.45% | 0.64% | 0.72% | 0.85% | 0.45% | 1.32% | 1.01% | 1.31% | 1.46% |
| 5 | 1-Hexanol | 3.84% | 4.19% | 2.58% | 2.65% | 3.71% | 3.59% | 6.58% | 6.22% | 5.01% | 4.52% |
| 6 | Isopentyl acetate | 3.05% | 3.38% | 2.85% | 3.07% | 5.21% | 9.68% | 5.10% | 4.07% | 3.23% | 8.26% |
| 7 | 4-Ethylbenzoic acid, 2 butylester | 0.00% | 0.00% | 0.19% | 0.28% | 7.69% | 10.50% | 14.34% | 12.04% | 3.35% | 3.36% |
| 8 | 1-heptanol | 0.00% | 0.00% | 0.00% | 0.00% | 0.38% | 0.30% | 0.00% | 0.00% | 0.35% | 0.32% |
| 9 | 1-octen-3ol | 0.00% | 0.00% | 0.00% | 0.00% | 0.07% | 0.07% | 0.03% | 0.07% | 0.05% | 0.02% |
| 10 | Ethyl hexanoate | 18.88% | 20.67% | 17.70% | 17.24% | 11.04% | 9.64% | 17.81% | 13.06% | 20.04% | 21.34% |
| 11 | Hexyl acetate | 0.29% | 0.30% | 0.26% | 0.31% | 0.10% | 0.31% | 0.18% | 0.10% | 0.11% | 0.10% |
| 12 | Limonene | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 13 | 2-ethyl hexanol | 0.22% | 0.34% | 0.11% | 0.09% | 0.46% | 0.27% | 0.15% | 0.13% | 0.14% | 0.10% |
| 14 | 4-Methyl benzaldehyde | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.10% | 0.00% | 0.00% | 0.00% | 0.00% |
| 15 | Octanol | 0.61% | 0.35% | 0.51% | 0.45% | 1.55% | 0.61% | 1.15% | 1.02% | 0.91% | 0.84% |
| 16 | 4-Ethylbenzaldehyde | 0.00% | 0.00% | 0.00% | 0.00% | 4.45% | 3.54% | 0.00% | 0.00% | 0.30% | 0.35% |
| 17 | 2-Phenyl ethyl alcohol | 22.79% | 24.21% | 24.33% | 25.96% | 19.82% | 22.55% | 15.85% | 25.12% | 21.99% | 18.97% |
| 18 | Diethyl succinate | 8.95% | 9.90% | 11.87% | 12.27% | 16.68% | 19.58% | 20.07% | 23.05% | 22.03% | 18.84% |
| 19 | Octanoic acid | 0.00% | 0.00% | 0.00% | 0.00% | 0.36% | 0.04% | 0.00% | 0.00% | 0.00% | 0.00% |
| 20 | Methyl salicylate | 0.00% | 0.00% | 0.04% | 0.03% | 5.19% | 3.57% | 0.04% | 0.04% | 0.04% | 0.07% |
| 21 | Ehtyl octanoate | 29.58% | 24.22% | 27.78% | 23.20% | 7.92% | 7.59% | 7.49% | 4.81% | 13.25% | 13.69% |
| 22 | Benzenacetiacid ethyl ester | 0.00% | 0.00% | 0.08% | 0.08% | 0.07% | 0.08% | 0.11% | 0.29% | 0.11% | 0.16% |
| 23 | 2-Phenylethylacetate | 0.57% | 0.77% | 0.71% | 0.80% | 0.75% | 1.51% | 1.33% | 1.38% | 0.51% | 0.56% |
| 24 | Ethyl decanoate | 7.77% | 8.32% | 7.26% | 9.32% | 3.12% | 2.27% | 1.52% | 1.21% | 3.00% | 2.94% |
| 25 | Ethyl dodecanoate | 0.15% | 0.09% | 0.46% | 0.51% | 0.77% | 0.52% | 0.19% | 0.19% | 0.25% | 0.32% |
| 26 | Ethyl hexadecanoate | 0.16% | 0.21% | 0.32% | 0.36% | 1.07% | 1.00% | 0.11% | 0.08% | 0.37% | 0.55% |
| | Total abundance | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% |

**Table SI 1b.** Relative abundances of the volatile compounds at the five sampling times (T1, T2, T3, T4, T5) in St. Magdalener wines.

| | T1 | | T2 | | T3 | | T4 | | T5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| St Magdalener | Conv | SuperC | Conv | SuperC | Conv | SuperC | Conv | SuperC | Conv | SuperC |
| 1 Acetic acid | 0.00% | 0.00% | 0.00% | 0.00% | 0.67% | 0.76% | 1.69% | 1.45% | 0.27% | 0.40% |
| 2 Ethyl butanoate | 1.44% | 1.43% | 1.00% | 0.99% | 1.33% | 1.11% | 1.57% | 1.21% | 1.03% | 1.35% |
| 3 Butanoic acid, 2-methyl ethyl ester | 0.24% | 0.22% | 0.20% | 0.21% | 0.34% | 0.22% | 0.31% | 0.31% | 0.27% | 0.41% |
| 4 Butanoic acid, 3-methyl ethyl ester | 0.40% | 0.37% | 0.34% | 0.34% | 0.71% | 0.46% | 0.56% | 0.54% | 0.55% | 0.87% |
| 5 1-Hexanol | 4.01% | 4.09% | 2.87% | 3.20% | 4.53% | 4.56% | 6.52% | 6.40% | 3.78% | 4.70% |
| 6 Isopentyl acetate | 6.47% | 6.08% | 5.67% | 5.67% | 14.19% | 18.24% | 7.68% | 8.13% | 16.46% | 12.16% |
| 7 4-Ethylbenzoic acid, 2 butylester | 0.19% | 0.13% | 0.31% | 0.21% | 7.04% | 7.82% | 9.92% | 11.37% | 0.00% | 1.64% |
| 8 1-heptanol | 0.28% | 0.28% | 0.00% | 0.00% | 0.37% | 0.37% | 0.00% | 0.00% | 0.00% | 0.17% |
| 9 1-octen-3ol | 0.00% | 0.00% | 0.00% | 0.00% | 0.19% | 0.12% | 0.04% | 0.03% | 0.02% | 0.01% |
| 10 Ethyl hexanoate | 13.98% | 13.68% | 12.33% | 13.47% | 13.29% | 11.46% | 12.75% | 11.77% | 12.71% | 16.89% |
| 11 Hexyl acetate | 0.98% | 1.55% | 0.97% | 0.95% | 0.47% | 0.48% | 0.64% | 0.53% | 0.43% | 0.26% |
| 12 Limonene | 0.12% | 0.13% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 13 2-ethyl hexanol | 0.09% | 0.11% | 0.00% | 0.06% | 0.23% | 0.25% | 0.11% | 0.11% | 0.08% | 0.11% |
| 14 4-Methyl benzaldehyde | 0.00% | 0.00% | 0.00% | 0.00% | 0.14% | 0.11% | 0.00% | 0.00% | 0.00% | 0.00% |
| 15 Octanol | 0.00% | 0.15% | 0.00% | 0.30% | 0.58% | 0.51% | 1.06% | 1.00% | 0.71% | 0.84% |
| 16 4-Ethylbenzaldehyde | 0.00% | 0.00% | 0.00% | 0.00% | 4.76% | 3.67% | 0.00% | 0.00% | 0.39% | 0.30% |
| 17 2-Phenyl ethyl alcohol | 28.62% | 23.06% | 22.79% | 22.90% | 14.20% | 12.69% | 16.92% | 17.17% | 19.57% | 16.86% |
| 18 Diethyl succinate | 15.21% | 12.46% | 13.02% | 13.62% | 11.82% | 11.43% | 25.27% | 24.98% | 26.34% | 24.05% |
| 19 Octanoic acid | 0.00% | 0.00% | 0.22% | 0.11% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 20 Methyl salicylate | 0.00% | 0.00% | 0.00% | 0.00% | 5.38% | 4.46% | 0.00% | 0.00% | 0.00% | 0.04% |
| 21 Ehtyl octanoate | 19.41% | 24.68% | 28.32% | 25.31% | 13.79% | 15.44% | 8.83% | 8.18% | 11.65% | 12.55% |
| 22 Benzenacetiacid ethyl ester | 0.06% | 0.06% | 0.05% | 0.06% | 0.03% | 0.00% | 0.19% | 0.22% | 0.10% | 0.14% |
| 23 2-Phenylethylacetate | 2.31% | 2.19% | 1.79% | 2.00% | 1.57% | 0.86% | 3.56% | 3.96% | 1.20% | 1.38% |
| 24 Ethyl decanoate | 5.71% | 8.68% | 9.00% | 9.46% | 2.78% | 3.72% | 1.68% | 1.94% | 4.09% | 3.96% |
| 25 Ethyl dodecanoate | 0.31% | 0.46% | 0.74% | 0.80% | 0.63% | 0.40% | 0.14% | 0.13% | 0.18% | 0.33% |
| 26 Ethyl hexadecanoate | 0.18% | 0.20% | 0.39% | 0.33% | 0.97% | 0.87% | 0.56% | 0.59% | 0.18% | 0.58% |
| Total abundance | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% |

**Table SI 1c**. Relative abundances of the volatile compounds at the five sampling times (T1, T2, T3, T4, T5) in Lagrein red wines.

| Lagrein | T1 | | T2 | | T3 | | T4 | | T5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Conv | SuperC | Conv | SuperC | Conv | SuperC | Conv | SuperC | Conv | SuperC |
| 1 Acetic acid | 0.00% | 0.00% | 0.00% | 0.00% | 1.73% | 0.26% | 3.92% | 4.17% | 0.72% | 1.06% |
| 2 Ethyl butanoate | 1.73% | 1.60% | 1.64% | 1.58% | 1.60% | 1.95% | 1.81% | 1.10% | 1.50% | 1.62% |
| 3 Butanoic acid, 2-methyl ethyl ester | 0.24% | 0.23% | 0.32% | 0.30% | 0.16% | 0.20% | 0.24% | 0.15% | 0.22% | 0.25% |
| 4 Butanoic acid, 3-methyl ethyl ester | 0.38% | 0.37% | 0.39% | 0.35% | 0.33% | 0.65% | 0.43% | 0.35% | 0.56% | 0.48% |
| 5 1-Hexanol | 3.72% | 4.00% | 3.39% | 3.43% | 4.48% | 4.64% | 4.71% | 4.37% | 3.10% | 4.37% |
| 6 Isopentyl acetate | 3.86% | 3.99% | 3.89% | 3.85% | 8.55% | 12.48% | 4.03% | 4.01% | 6.13% | 11.23% |
| 7 4-Ethylbenzoic acid, 2 butylester | 0.00% | 0.00% | 1.03% | 0.04% | 8.54% | 6.55% | 12.36% | 11.23% | 1.36% | 4.38% |
| 8 1-heptanol | 0.00% | 0.00% | 0.00% | 0.00% | 0.35% | 0.39% | 0.00% | 0.00% | 0.11% | 0.12% |
| 9 1-octen-3ol | 0.00% | 0.10% | 0.00% | 0.00% | 0.12% | 0.13% | 0.03% | 0.03% | 0.01% | 0.02% |
| 10 Ethyl hexanoate | 13.94% | 14.91% | 14.72% | 14.11% | 8.29% | 12.47% | 10.35% | 9.77% | 13.43% | 14.38% |
| 11 Hexyl acetate | 0.62% | 0.60% | 0.58% | 0.56% | 0.34% | 0.49% | 0.32% | 0.24% | 0.33% | 0.25% |
| 12 Limonene | 0.07% | 0.06% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 13 2-ethyl hexanol | 0.16% | 0.12% | 0.00% | 0.00% | 0.19% | 0.22% | 0.05% | 0.06% | 0.07% | 0.08% |
| 14 4-Methyl benzaldehyde | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 15 Octanol | 3.07% | 0.50% | 0.49% | 0.42% | 0.73% | 0.51% | 0.90% | 0.17% | 0.64% | 0.55% |
| 16 4-Ethylbenzaldehyde | 0.00% | 0.00% | 0.00% | 0.00% | 4.24% | 2.05% | 0.00% | 0.00% | 0.28% | 0.19% |
| 17 2-Phenyl ethyl alcohol | 19.06% | 18.10% | 17.05% | 18.80% | 11.57% | 11.34% | 14.87% | 24.92% | 18.79% | 12.51% |
| 18 Diethyl succinate | 15.81% | 16.08% | 20.26% | 19.87% | 29.61% | 16.61% | 34.83% | 29.72% | 30.35% | 30.79% |
| 19 Octanoic acid | 0.00% | 0.00% | 0.00% | 0.00% | 0.10% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 20 Methyl salicylate | 0.00% | 0.00% | 0.00% | 0.00% | 5.91% | 3.08% | 0.00% | 0.00% | 0.00% | 0.00% |
| 21 Ehtyl octanoate | 27.87% | 28.90% | 26.89% | 26.46% | 8.17% | 19.75% | 6.86% | 6.11% | 14.18% | 11.74% |
| 22 Benzenacetiacid ethyl ester | 0.09% | 0.09% | 0.13% | 0.12% | 0.25% | 0.03% | 0.17% | 0.13% | 0.12% | 0.09% |
| 23 2-Phenylethylacetate | 0.75% | 0.67% | 0.97% | 0.94% | 1.37% | 0.67% | 1.15% | 0.98% | 0.80% | 0.47% |
| 24 Ethyl decanoate | 7.99% | 8.94% | 7.31% | 7.90% | 2.03% | 4.62% | 2.07% | 2.20% | 6.63% | 4.92% |
| 25 Ethyl dodecanoate | 0.46% | 0.49% | 0.56% | 0.90% | 0.61% | 0.40% | 0.77% | 0.16% | 0.36% | 0.29% |
| 26 Ethyl hexadecanoate | 0.18% | 0.24% | 0.37% | 0.38% | 0.72% | 0.50% | 0.14% | 0.11% | 0.30% | 0.23% |
| Total abundance | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% |

**Table SI 1d**. Relative abundances of the volatile compounds at the five sampling times (T1, T2, T3, T4, T5) in Lagrein rosé wines.

| | T1 | | T2 | | T3 | | T4 | | T5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lagrein Rosè | Conv | SuperC | Conv | SuperC | Conv | SuperC | Conv | SuperC | Conv | SuperC |
| 1 Acetic acid | 0.00% | 0.00% | 0.23% | 0.00% | 0.43% | 0.36% | 1.01% | 1.11% | 0.00% | 0.08% |
| 2 Ethyl butanoate | 1.09% | 1.22% | 0.84% | 0.88% | 1.71% | 2.37% | 2.17% | 1.16% | 0.97% | 1.12% |
| 3 Butanoic acid, 2-methyl ethyl ester | 0.03% | 0.03% | 0.04% | 0.04% | 0.06% | 0.07% | 0.06% | 0.04% | 0.05% | 0.04% |
| 4 Butanoic acid, 3-methyl ethyl ester | 0.06% | 0.07% | 0.05% | 0.07% | 0.15% | 0.18% | 0.16% | 0.11% | 0.13% | 0.12% |
| 5 1-Hexanol | 2.15% | 2.40% | 1.85% | 2.57% | 5.26% | 6.61% | 5.67% | 5.72% | 2.72% | 3.16% |
| 6 Isopentyl acetate | 24.90% | 26.88% | 16.97% | 29.41% | 28.00% | 32.27% | 25.67% | 25.10% | 15.87% | 15.28% |
| 7 4-Ethylbenzoic acid, 2 butylester | 0.00% | 0.00% | 0.00% | 0.00% | 4.45% | 4.04% | 2.98% | 3.12% | 0.00% | 0.00% |
| 8 1-heptanol | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 9 1-octen-3ol | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 10 Ethyl hexanoate | 11.62% | 11.75% | 12.35% | 12.87% | 18.89% | 21.20% | 16.17% | 16.46% | 16.42% | 16.95% |
| 11 Hexyl acetate | 7.80% | 7.39% | 7.03% | 7.16% | 4.46% | 4.87% | 4.67% | 4.32% | 4.19% | 3.74% |
| 12 Limonene | 0.04% | 0.04% | 0.02% | 0.02% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |
| 13 2-ethyl hexanol | 0.02% | 0.03% | 0.01% | 0.01% | 0.10% | 0.18% | 0.03% | 0.03% | 0.00% | 0.00% |
| 14 4-Methyl benzaldehyde | 0.00% | 0.00% | 0.00% | 0.00% | 0.11% | 0.11% | 0.00% | 0.00% | 0.00% | 0.00% |
| 15 Octanol | 0.05% | 0.07% | 0.05% | 0.06% | 0.12% | 0.13% | 0.07% | 0.09% | 0.04% | 0.07% |
| 16 4-Ethylbenzaldehyde | 0.00% | 0.00% | 0.00% | 0.00% | 7.53% | 5.48% | 0.00% | 0.00% | 0.13% | 0.25% |
| 17 2-Phenyl ethyl alcohol | 4.21% | 4.64% | 3.86% | 4.04% | 4.57% | 3.45% | 8.19% | 8.72% | 2.20% | 2.47% |
| 18 Diethyl succinate | 1.19% | 1.35% | 1.49% | 1.89% | 5.05% | 4.03% | 7.25% | 7.37% | 3.69% | 4.68% |
| 19 Octanoic acid | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.69% | 0.65% | 0.31% | 0.36% |
| 20 Methyl salicylate | 0.00% | 0.00% | 0.00% | 0.00% | 6.18% | 3.31% | 0.00% | 0.00% | 0.00% | 0.00% |
| 21 Ehtyl octanoate | 34.63% | 33.23% | 39.42% | 31.13% | 3.04% | 2.91% | 18.62% | 18.76% | 51.67% | 49.80% |
| 22 Benzenacetiacid ethyl ester | 0.02% | 0.02% | 0.03% | 0.03% | 0.12% | 0.10% | 0.05% | 0.06% | 0.05% | 0.05% |
| 23 2-Phenylethylacetate | 1.75% | 1.43% | 2.12% | 2.14% | 3.25% | 3.03% | 2.67% | 2.90% | 1.17% | 1.40% |
| 24 Ethyl decanoate | 10.14% | 9.20% | 12.88% | 7.11% | 4.86% | 3.50% | 3.72% | 4.02% | 0.00% | 0.00% |
| 25 Ethyl dodecanoate | 0.18% | 0.12% | 0.52% | 0.45% | 0.61% | 0.81% | 0.07% | 0.09% | 0.25% | 0.25% |
| 26 Ethyl hexadecanoate | 0.11% | 0.10% | 0.21% | 0.13% | 1.04% | 0.99% | 0.09% | 0.17% | 0.15% | 0.19% |
| Total abundance | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% | 100.00% |

# CHAPTER 3

# Qualitative study of the binding properties of the Novel Cyclic Proanthocyanidins to the wine metals

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Part of the work described in this chapter has been previously published in the following scientific journals

Journal of The American Society for Mass Spectrometry
doi.org/10.1007/s13361-017-1876-8


Journal of The American Society for Mass Spectrometry
doi.org/10.1007/s13361-018-2044-5


Journal of Mass Spectrometry
doi.org/10.1002/jms.4280


Rapid Communication in Mass Spectrometry
doi.org/10.1002/rcm.8221

## 3.1 Introduction

The prevention of the precipitation of tartrate salts in bottled wine is a fundamental oenological technique applied to preserve wine from the associated turbidity (Lasanta *et al.,* 2012). In fact, potassium hydrogen tartrate (KHT) is usually present at supersaturated concentrations in wines and it leads often to precipitation of crystals. For example, the saturation point of KHT at 20°C in a 10% ethanol solution is 2.9 g/L (Ribéreau-Gayon *et al.,* 2006). In reality, KHT is present at higher concentrations in wine. Therefore, precipitation is thermodynamically favored at the temperatures at which wine is usually stored. In addition, the much less soluble calcium tartrate can precipitate, but still its concentration is much lower than KHT in wine. Calcium is often introduced incidentally, for example as bentonites (aluminum silicates hydrated with $Ca^{2+}$ and $Na^{+}$ counter-cations) which are used to prevent protein haze formation. Several approaches have been adopted in order to avoid the overtime KHT crystallization. An effective one is the addition of polymers able to provide protecting colloids, such as metatartaric acid or carboxymethyl cellulose (Ribéreau-Gayon *et al.,* 2006). When KHT aggregates in the presence of metatartaric acid it tends to include the polymer in the growing crystals. This prevents the crystal growth and the precipitation. Carboxymethyl cellulose has been shown to be more effective in prolonging the stability of wine, since metatartaric acid can be further hydrolysed to tartaric acid units, this way further increasing the probability of precipitation instead. Another strategy is cold treatment at temperatures close to the wine freezing point. This would force the salts above the saturation level to precipitate, therefore reducing the presence of long-term precipitation by direct filtration of the excess salt precipitate. Other approaches have been proposed, such as resin ion exchange (e.g. $K^{+}$, $Ca^{2+}$ and $Mg^{2+}$ for $H^{+}$ or $Na^{+}$) or increasing the quantity of mannoproteins dissolution into wine from spent yeasts through the *batonnage* technique (Gonçalves *et al.,* 2002; Núñez *et al.,* 2006; Giovani *et al.,* 2007; Diako *et al.,* 2016).

Besides, proanthocyanidins (PACs) are among those abundant polyphenol components of wine that have been shown to prevent tartrate instability (Ribéreau-Gayon *et al.,* 2006).

### *3.1.1 Novel cyclic proanthocyanidins disambiguation*

Proanthocyanidins (PAC) are a class of oligomeric flavonoids widespread in plants (Guyot *et al.,* 1997; Prior *et al.,* 2001; Vivas *et al.,* 2001b; Yu *et al.,* 2006; Robbins *et al.,* 2009; Falleh *et al.,* 2011; Sui *et al.,* 2016; Hollands *et al.,* 2017); in grapes they .are present in skin and seeds (Bordiga *et al.,* 2011; He *et al.,* 2008; Souquet *et al.,* 1996; Castañeda-Ovando *et al.,* 2009). They are particularly abundant in derived food products and beverages, where they play a major role as antioxidants with consequent beneficial effects on shelf-life and health (Mulero *et al.,* 2009; EFSA, 2011; Ceymann *et al.,* 2012; Ayoub *et al.,* 2015; Shahidi *et al.,* 2015). Their molecular structures are composed of linked flavan-3-ol monomers. Procyanidins are an important subclass composed only of (+)-catechin and (–)-epicatechin. In recent pioneering reports, the presence of tetrameric and pentameric novel cyclic PAC (therein referred as crown proanthocyanidins) in red wines from the Bordeaux region was proposed for the first time (Jourdes *et al.,* 2016; Jouin *et al.,* 2017). Ongoing investigation on the presence of oligomers in wines from this and other geographical regions continues to uncover new species such as the recently reported cyclic hexameric procyanidin (Longo *et al.,* 2018a; 2018b). The parallel identification of the same cyclic procyanidins in cranberries and peanuts skin was also disclosed (Longo *et al.,* 2018a; 2018b). MS and NMR characterizations of the tetrameric and pentameric novel cyclic PAC from purified extracts were provided. Despite the thorough characterization performed, this observation (the cyclic 'crown' structure of these PAC) was affected by an unforeseen ambiguity: the proposed novel cyclic B-type PAC share the same elemental composition with an already known class of (non-cyclic) PAC (seldom observed in wine) containing the additional A-type O-C linkage, usually present between the n and n-1 monomers in a n-mer (Li *et al.,* 2008; Gu *et al.,* 2003). An example of a compound of that non-cyclic PAC class in red wine is procyanidin A2 (Vivas *et al.,* 2001a*)*. In addition, this A-type PAC class up to the tetramer was reported to be abundant in cranberries (Prior *et al.,* 2001). The proposed novel cyclic B-type PAC and the non-cyclic A-type PAC possess identical elemental compositions. Therefore, the application of mass spectrometry for directly clarifying the actual structure of the new cyclic oligomers in wine may not be straightforward. $MS^n$

methods may also yield insufficient results since the A-type linkage can be broken during MS/MS fragmentation leaving only the B-type linkage (Li *et al.,* 2008; Gu *et al.,* 2003). It was noted, however, that these classes are distinct with respect to the number of phenolic protons they bear, and these are labile towards solvent exchange. An example of each of the different procyanidin classes (cyclic and non-cyclic, A- and B-types) is reported in Figure 35. The labile phenolic protons are indicated by circles; arrows also indicate the positions that cannot undergo solvent exchange because of the presence of an A-linkage.

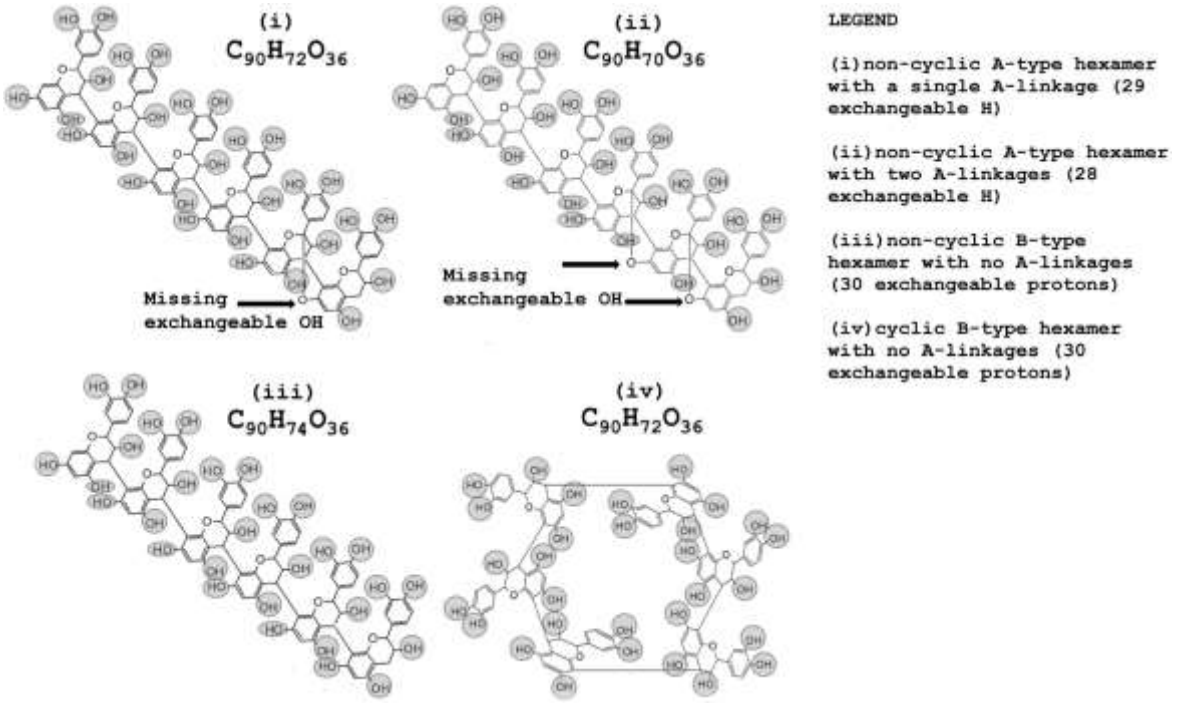


**Figure 35**. Models for the hexameric procyanidins. (i) Non-cyclic A-type hexameric procyanidin with one A linkage (isomeric form a); (ii) non-cyclic A-type hexameric procyanidin with two A-linkages; (iii) non-cyclic B-type hexameric procyanidin; (iv) cyclic B-type hexameric procyanidin with one head-tail B-linkage (isomeric form b). Neither the configurations of the stereogenic centers nor the C4-C6 inter-monomeric linkages preferences were resolved; the configurations shown are just examples.

In this respect, a direct approach supporting the structural NMR characterization was achieved by devising a chemical modification able to differentiate between the two different chemical bond arrangements of the two PAC classes (cyclic B-type and non-cyclic A-type). Among the most exploited derivatization approaches, (Quirke *et al.,* 1994; Lam *et al.,* 2002; Xu *et al.,* 2011; Qi *et al.,* 2014) hydrogen/deuterium (H/D) exchange offers a valuable option for structural resolution whenever exchangeable

protons are present. Moreover, the H/D exchange rates influence conformational mobility, hydrogen bonding strength, and solvent accessibility of the molecule studied (Olsen *et al.,* 2000; Liu *et al.,* 2005; Carlson *et al.,* 2007; Nemes *et al.,* 2008; Yan *et al.,* 2009). Nonetheless, this modification is completely reversible, and care must be applied to obtain a complete deuteration. Accordingly, HPLC methods have been modified for fully profiting from this transformation, for example by employing deuterium oxide as replacement of the water phase in HPLC-MS gradient methods (Olsen *et al.,* 2000). A critical step is the equilibration time for a complete H/D exchange, in particular for proteins, which is influenced by the substrate, the solution pH, and the temperature (Yan *et al.,* 2009). H/D exchange was infrequently but successfully applied to wine extracts to characterize anthocyanins and pyranoanthocyanins, demonstrating how the H/D exchange rate followed an apparent first order kinetic independent from the solution pH (Bakker *et al.,* 1997*)*. The application of this method showed that B-type species exchanged one H more than their correspondent A-type procyanidin analogues. Hence, these species are not isobaric after HDX, and they can be differentiated by mass-spectrometric detection alone, without resorting to NMR or other structural approaches. While HDX was successfully applied to the cyclic tetrameric candidate with the substitution of five positions per (epi)catechin monomer (m/z 1153.2608 $\{H_2O\}$ became m/z 1174.3926 $\{D_2O\}$), no evidence of its effect for the known A-type procyanidins could be obtained due to their scarcity in the analyzed matrices (wine), with an exception for some traces found in a cranberry (*Vaccinium* spp.) extract. This scarcity in cranberries was unexpected since earlier works had proposed the presence of the A-type procyanidin tetramer as a major component (Prior *et al.,* 2001). Other differences were noticed, namely the distribution of the retention times of the cyclic oligomers (eluting in HPLC earlier than their non-cyclic analogues) and their $MS^2$ spectra. During the MS/MS experiments, these cyclic oligomers showed much lower degrees of fragmentation than their linear analogues. Moreover, the cyclic analogues displayed a much more limited isomeric distribution, with only one main isomer in contrast to the ample variability of their linear analogues. This was already demonstrated by NMR studies on the tetramer (Jourdes *et al.,* 2016). It was argued that strict requirements may be needed for the cyclization of the non-cyclic precursor, and this

may cause the observed limitation in the distribution of cyclic isomers. The macromolecular conformations depend on the specific stereogenic configurations of the carbon atoms (i.e., number and relative bindings of (+)-catechins and (–)-epicatechins) as well as on the specific interflavan-3-ol binding preferences (C4-C6 or C4-C8). Thus, only specific arrangements of monomers may lead to cyclization. It was also confirmed that in general, the cyclic analogues elute at lower retention times in reversed-phase LC, indicating that they possess a higher polarity than most of their non-cyclic analogues. Also, this effect may be a consequence of the macromolecular conformation of these species. After the confirmation for the cyclic B-type tetramer (Longo *et al.*, 2018a), a proper validation of the HDX method on a matrix rich in A-type procyanidins was performed. In this purpose, peanut skin was selected as a rich source of A-type procyanidins. In fact, peanut skin was known for containing dimeric, trimeric, tetrameric, and pentameric A-type procyanidins in high proportions (Yu *et al.,* 2006). This further investigation allowed for unambiguous identification of A-type procyanidins in peanut skin and B-type (cyclic and non-cyclic) procyanidins in wine for the first time. In fact, previous study did not provide a full identification of pentameric and hexameric B-type cyclic PCA (Longo *et al.,* 2008a).

Hence, hydrogen/deuterium exchange provided a direct method to support the structural resolution of unconventional PAC in red wine cranberry extract and peanut skin. With this technique, it was demonstrated that the main, most abundant PAC tetramer (referring to m/z 1153.2608, eluting at 3.9 min) in wine belonged to the proposed novel class of cyclic oligomeric B-type PAC. Moreover, this compound was identical (same retention times, exact masses, and MS/MS fragmentations) to the major tetrameric PAC found in cranberries. Traces of A-type LTP were, however, observed in cranberries and (much less) in wine at later retention times, but they were negligible with respect to the B-type CTP in both samples. Moreover, B-type cyclic pentamers and hexamers in wine have been elucidated with hydrogen/ deuterium exchange (HDX) for the first time. This confirmed the usefulness of HDX for structural investigations on cyclic procyanidins complementary to NMR and other structural analytical techniques.

### *3.1.2 Aim of the study*

The ability of phenolic antioxidants and polyphenols to bind metals has been studied (Satterfield *et al.,* 2000; Davis *et al.,* 2004; Davis *et al.,* 2005; Zhang *et al.,*2005a; 2005b; Davis *et al.,* 2006; Pikulski *et al.,* 2007; Kiefer *et al.,* 2008; Wang *et al.,* 2009; Danilewicz, 2013). The recent discovery of the novel cyclic PAC have raised interest in their chemical properties. The aim of the present study is a preliminary qualitative investigation of the binding properties of these novel PACs to metals in several red and white wines. Mass spectrometry is an established method for the analysis of metal-polyphenol complexes. However, methods involving reversed-phase liquid chromatography (RP-LC) separations prior to the MS analysis are not employed extensively for the elucidation of such complexes (Fernandez *et al.,* 2002; Pikulski *et al.,* 2007) Nonetheless, the aim of this work is far from the estimation of binding constants or geometries, but is instead a qualitative assessment of the presence of preferential bindings. The use of MS coupled to LC, although avoided usually for these metal complexes, (Davis *et al.,* 2004; Pikulski *et al.,* 2007) could allow to ascertain (only qualitatively) the hypothesis that the different isomers may exert different affinities for potassium and calcium. Furthermore, the identification of new chemical markers of wine quality and authenticity is also a main aim of this investigation. The wide variety of these species studied in recent reports (Longo *et al.*, 2018c) allowed the proposal that their relative abundances could be used as tools for differentiating the wines by grape variety. Another sought application would be the definition of suitable variables for monitoring the effects of technological applications, as for example the use of metatartaric stabilization and the use bentonite.

## 3.2 Material and Methods

### *3.2.1 Material*

All solvents and additives used (LC-MS grade) were purchased from Sigma-Aldrich Ltd. Wines from South Tyrol (Italy) were collected in a local winery (Kellerei Bozen/Bolzano, BZ, Italy) and an agricultural high school (Happacherhof, Auer/Ora, BZ, Italy). The wines were all of PDO/DOC grade (Table 9).

**Table 9.** List of studied wines

| SAMPLE | WINE NAME | ABBREVIATION |
|---|---|---|
| **6** | Lagrein | L |
| **7** | Lagrein Prestige Klebelsberg | LP |
| **8** | Lagrein Eyrl | LE |
| **9** | Lagrein Grieser Collection I | LG-1 |
| **16** | Lagrein Grieser, Collection 2 | LG-2 |
| **2** | Cabernet Franc | CF |
| **3** | Cabernet Sauvignon | CS |
| **4** | Merlot collection | MC |
| **5** | Merlot barrique | MB |
| **10_1** | Blauburgunder | BB |
| **10_2** | Blauburgunder | BB-rep |
| **1** | St.Magdalener Moar | SMM |
| **17** | St.Magdalener Classico Huck-I | SMH-1 |
| **18** | St.Magdalener classico Huck-II | SMH-2 |
| **11** | Gewürztraminer Kleinstein | GK |
| **12** | Gewürztraminer | G |
| **14** | Gewürztraminer Passito | GP |
| **13** | Sauvignon Blanc | SB-1 |
| **15** | Sauvignon Blanc | SB-2 |
| **19** | Aurum, Chardonnay Passito | Au |

### *3.2.2 Preparation of samples*

No extraction was applied to the samples. They were instead concentrated at reduced pressure (9-10 mbar) at 30°C. Then, they were dried by 30 min of gentle $N_2$ flux. Finally, they were recovered in the mobile phase A (see HPLC-HRMS section 3.2.3 for details) to a final concentration 10-fold higher than the initial one. For the MS/MS studies, the recovery was limited in order to provide a concentration 10 to 30-fold higher. The samples were always filtered before column injection (0.2 µm, regenerate cellulose).

### *3.2.3 HPLC/HRMS/MS analysis*

The HPLC/HRMS/MS method applied was presented earlier (Longo *et al.,* 2017; Savini *et. al.,* 2017; Longo *et. al.,* 2018a). Briefly, the HPLC/HRMS system was

composed of a Q Exactive HRMS instrument (Thermo Fisher Scientific, Rodano, Milano, Italy) coupled with a 16-channel DAD-provided Agilent 1260 HPLC (Agilent Technologies Itala S.p.A., Cernusco sul Naviglio, Milano, Italy). The separation was carried out at 1 mL/min on a ODS Hypersil C18 column (125 mm × 4.6 mm i.d., 5 µm, Thermo Sci.) equipped with a pre-column filter (ODS Hypersil, 5 µm pore size, 10 x 4 mm drop-in guards, Thermo Fisher Scientific). The mobile phase consisted of a combination of solvent A (0.1% v/v formic acid in 0.02 mol/L ammonium formate in water) and B (0.1% v/v formic acid in saturated ammonium formate acetonitrile LC/MS grade). The gradient was set as follows: from 5% B at 0 min to 25% B (v/v) at 21 min, then to 95% B at 22 min until 27 min, to 5% at 28 min, followed by re-equilibration step (5% B) at 32 to 35 min. The DAD spectra were recorded from 210 to 600 nm and provided real-time monitoring at 280 nm, 320 nm, 365 nm, 420 nm and 520 nm (+/- 4 nm). The Q Exactive HESI source was operated in positive ionization mode using the following conditions: sheath gas = 20 (arbitrary units), aux gas, 5 (arbitrary units), aux temperature, 250 °C, spray voltage, +3.5 kV, capillary temperature, 320 °C and RF S-lens, 70. The mass range selected was from 500 to 2000 m/z with a Full-MS working resolution = 70000 (@200 m/z), AGC target, $3\times10^6$, max. injection time, 300ms. Data dependent HPLC/MS/MS experiments were run separately on the $N_2$ concentrated samples: Full-MS parameters were kept as shown, MS/MS AGC, $10^6$, max. injection time, 300; FT-MS set resolution = 35000; loop count, 5; isolation window, 2 or 3 m/z with 1 m/z offset; normalized collision energy, 15 eV. For data dependent settings: minimum AGC target, $3\times10^{-3}$; apex trigger, 2-8 s; charge exclusion, 3–8; and higher, dynamic exclusion, 3 s. LC/MS/MS experiments were tested also in negative ionization (spray voltage -3.5 kV). Lock masses were constantly employed to correct mass deviations across the Full-MS acquisition range throughout the experiments. The HPLC/DAD data were collected and analyzed by OpenLab software while the MS data and results were collected and analyzed by Xcalibur 3.1 software (Thermo Fisher Scientific). XLStat (version 2016.02.28430, Addinsoft, Paris, France) was employed for the statistical analysis and The Unscrambler (version 10.4.43636.111, CAMO Software AS., Oslo, Norway) software were employed for the statistical analysis.

## 3.3 Results and Discussion

The 19 wines are reported in Table 9. They were analyzed for the presence of the metal adducts of proanthocyanidins, from dimeric to hexameric oligomers. The main cations investigated were potassium and calcium as their concentrations are important variable in wine. In addition, magnesium, zinc, lead, cadmium, iron and copper were however investigated but the re-concentration (ten times the native concentration) of wine was not yet suitable for their identification therefore no results are reported about them. Sodium adducts instead were observed and included in the list. In Table 10 the metal adducts identified are reported.

**Table 10** List of metal-PAC adducts identified in wines from South-Tyrol. Corresponding shown MS spectra in SI are from sample 10.

| Species | Elemental Composition | Found Mass (m/z) | δ (ppm) | R.time(s) (±0.1 min) | Figure | Comments |
|---|---|---|---|---|---|---|
| dimer-$K^+$ | $[C_{30}H_{26}KO_{12}]^+$ | 617.1053 | -0.5 | 4.2, 5.0, 7.6, 9.4, 10.3 | **SI1** | eluting at most of the EIC r.t. of the main dimer-$H^+$ species |
| dimer-$Na^+$ | $[C_{30}H_{26}NaO_{12}]^+$ | 601.1328 | 2.0 | 7.8, 10.6, 16.3, 23.6, 23.8 | **SI10** | eluting at most of the EIC r.t. of the main dimer-$H^+$ species. The most intense at the highest r.t. |
| trimer-$K^+$ | $[C_{45}H_{38}KO_{18}]^+$ | 905.1683 | 0.7 | 3.3 | **SI2** | eluting only at the EIC r.t. of the more polar trimer-$H^+$ species |
| trimer-$Na^+$ | $[C_{45}H_{38}NaO_{18}]^+$ | 889.1967 | 1.9 | 1.6, 23.8 | **SI10** | eluting mostly at the latest EIC r.t. of the main dimer-$H^+$ species |
| l-tetramer-$K^+$ | $[C_{60}H_{50}KO_{24}]^+$ | 1193.2338 | 1.2 | 1.0, 3.3, 3.9, 4.4, 7.4, 8.6, 9.4 | **SI3** | only weak traces eluting at much anticipated r.t. than the correspondent main l-tetramer-$H^+$ species |
| l-tetramer-$Na^+$ | $[C_{60}H_{50}NaO_{24}]^+$ | 1177.2593 | 0.8 | 9.4, 11.4, 23.7 | **SI10** | eluting at some of the EIC r.t. of the main dimer-$H^+$ species and at 23.7 min as all the other $Na^+$ adducts |
| l-tetramer-l-galloc-$Ca^{2+}$ | $[C_{60}H_{50}CaO_{25}]^{2+}$ | 605.1134 (z = +2) | 1 | 3.9 4.3, 10.2 | **SI4** | not all isobaric peaks are present in all the samples |

| | | | | | | |
|---|---|---|---|---|---|---|
| c-tetramer-$K^+$ | $[C_{60}H_{48}KO_{24}]^+$ | 1191.2160 | -0.6 | 3.7 | **SI5** | eluting at the same r.t. of the correspondent main c-tetramer-$H^+$ species. Absence of higher r.t. traces (associated to the a-type tetramer) |
| c-tetramer-$Ca^{2+}$ | $[C_{60}H_{48}CaO_{24}]^{2+}$ | 596.1072 (z = +2) | -0.5 | 1.0,1.7, 6.4, 9.4, 11.0 | **SI6** | much wider distribution of r.t. than the corresponding c-tetramer-$H^+$ species |
| c-tetramer-l-galloc-$K^+$ | $[C_{60}H_{48}KO_{25}]^+$ | 1207.2111 | -0.4 | 2.5 | **SI7** | eluting at the same r.t of the correspondent c-tetramer-l-galloc-$H^+$ |
| c-tetramer-l-galloc-$Ca^{2+}$ | $[C_{60}H_{48}CaO_{25}]^{2+}$ | 604.1064 (z = +2) | 1.8 | 2.8, 3.9, 4.3, 5.3, 14.8 | **SI8** | traces |

The relative abundances obtained for each PAC in all 19 samples are reported in Table SI1 (a-d) Supporting Information). Firstly, three factors accounted for the distribution of these species in the samples: i) the total abundance of the specific ions; ii) the total abundance of the specific oligomer; iii) the affinity of the oligomers for the ions. Another interesting fact was the affinity on some metal for specific congeners (e.g. trimer-$K^+$). The PC trimer complex with potassium appeared to occur only with one trimer congener eluting at 3.3 min. This same effect was not observed for the dimer, since the correspondent dimer-$K^+$ adduct eluted in correspondence to the dimer-$H^+$ adduct. This evidence indicates that the affinity of the trimer with potassium is not possibly only due to the number of monomer groups and it should depend instead on certain preferred conformations of the macromolecule, which is induced by the specific linkages of (+)-catechins and (-)-epicatechins and their mutual binding (C4-C6 vs C4-C8) and ratio and location of (+)-catechins and (-)-epicatechins. The other trimer-$H^+$ isomers, although similar in intensity, did not display an associated $K^+$-adduct. Interestingly, this congener was precisely the most polar one among trimers (i.e. the first one eluting at 3.3 min). However, this effect appears to be limited to this one instance. Besides, other oligomer showed co-elution of certain adducts with their $[M-H]^+$ analogues. This was common for most of the identified adducts but not for all of them. The origin of these adducts in wine itself (and not artefacts formed during the analysis) is confirmed since not all wine samples possessed all adducts or in the

same proportions (see Table SI 1 in Supporting Information): this excludes the formation of adducts during the elution (also, LC-MS grade solvents were used). Moreover, it is interesting to note that these complexes must be relatively stable. In fact, the eluent had a relatively high concentration (phase A: 0.02 mol/L, phase B: saturated) of ammonium formate salt. Its ionic strength may displace other bound cations. Moreover, as exposed previously, the binding must be conformation-selective since the relative abundances were strongly influenced by the specific conformations and not by the number of catechol units only. This was confirmed by comparison between the cyclic and the linear oligomers (from the tetramer upwards). Whereas the proportion of cyclic procyanidins and cyclic prodelphinidins varied according to the grape variety used for wine, here the preferences for potassium and calcium complex formation had totally different profiles. For instance, in almost all wines, calcium bound the cyclic tetramer procyanidin almost 100% over the total (cyclic plus non-cyclic) tetramer procyanidins. Similarly, potassium appeared to favour entirely the cyclic over the non-cyclic pentamer in four red wines, namely Lagrein, Merlot, Pinot Noir and two St.Magdalener but quite the opposite for all white wines, namely Gewürztraminer, Sauvignon and Chardonnay. In addition, sodium bound exclusively to the linear tetramer, and not the cyclic one. Statistical analysis was then applied to investigate the contribution of these variables (the relative abundances of metal-PAC adducts as measured with the current method) to the total variance. Those variables most significant for the sample variance were selected by ANOVA and listed in Table 11.

**Table 11** ANOVA (for all observations): Means for variable Variety. All and only the significant variable are shown.

| Species | Index used in tables and charts | Pr > F |
|---|---|---|
| **l-Dimer +$H^+$** | 579 | 0.000 |
| **l-Dimer +$K^+$** | 617 | 0.001 |
| **l-Trimer +$H^+$** | 867 | 0.000 |
| **l-Trimer +$K^+$** | 905 | 0.000 |
| **l-Trimer +$Na^+$** | 889 | 0.002 |
| **l-Tetramer +$H^+$** | 1155 | 0.000 |
| **l-Tetramer +$K^+$** | 1193 | 0.010 |
| **l-Tetramer +$Na^+$** | 1177 | 0.003 |

| | | |
|---|---|---|
| **c-Tetramer $+H^+$** | 1153 | 0.000 |
| **c-Tetramer $+K^+$** | 1191 | 0.026 |
| **l-Pentamer $+H^+$** | 1443 | 0.000 |
| **l-Pentamer $+K^+$** | 1481 | |
| **l-Pentamer $+Ca^{2+}$** | 741 | 0.000 |
| **c-Pentamer $+H^+$** | 1441 | 0.000 |
| **l-Hexamer $+H^+$** | 1731 | 0.000 |
| **l-Hexamer $+K^+$** | 1769 | |
| **c-Hexamer $+Ca^{2+}$** | 884 | |
| **c-Hexamer $+H^+$** | 1729 | 0.000 |
| **c-Hexamer $+K^+$** | 1767 | |
| **l-Tetramer-1-OH $+H^+$** | 1171 | 0.000 |
| **c-Tetramer-1-OH $+H^+$** | 1169 | 0.000 |
| **c-Tetramer-1-OH $+K^+$** | 1207 | 0.000 |
| **c-Tetramer-1-OH $+Ca^{2+}$** | 604 | 0.034 |
| **l-Pentamer-1-OH $+H^+$** | 1459 | 0.000 |
| **l-Pentamer-1-OH $+Ca^{2+}$** | 749 | 0.000 |
| **c-Pentamer-1-OH $+H^+$** | 1457 | 0.000 |
| **c-Pentamer-1-OH $+K^+$** | 1495 | 0.005 |
| **c-Pentamer-1-OH $+Ca^{2+}$** | 748 | 0.028 |

In Figure 36 and 37 the Principal Component Analysis for red and white wines respectively are shown, using the relative abundances of these species as variables is reported. The processing was done separately for white and red wines due to their heterogenous nature. The PCA separations of the wines by their grape variety worked less well here than in previous reports (where no metal complex variable had been applied; Longo *et al.,* 2018c); still a neat separation was achieved by grape variety for red and for white wines alike. A particular exception was the St.Magdalener samples. Two St.Magdalener (Huck am Back) were neatly separated from a third one (MOAR). This set of variables appeared to be less affected by the grape variety and some other effect (e.g. winemaking procedures) may be involved. In fact, these wines are produced by the same wineries using grapes from different vineyards (but geographically close). Other variables employed previously did not show any significant specific separation for these samples in PCA of cluster analysis (Longo *et al.,* 2018c).

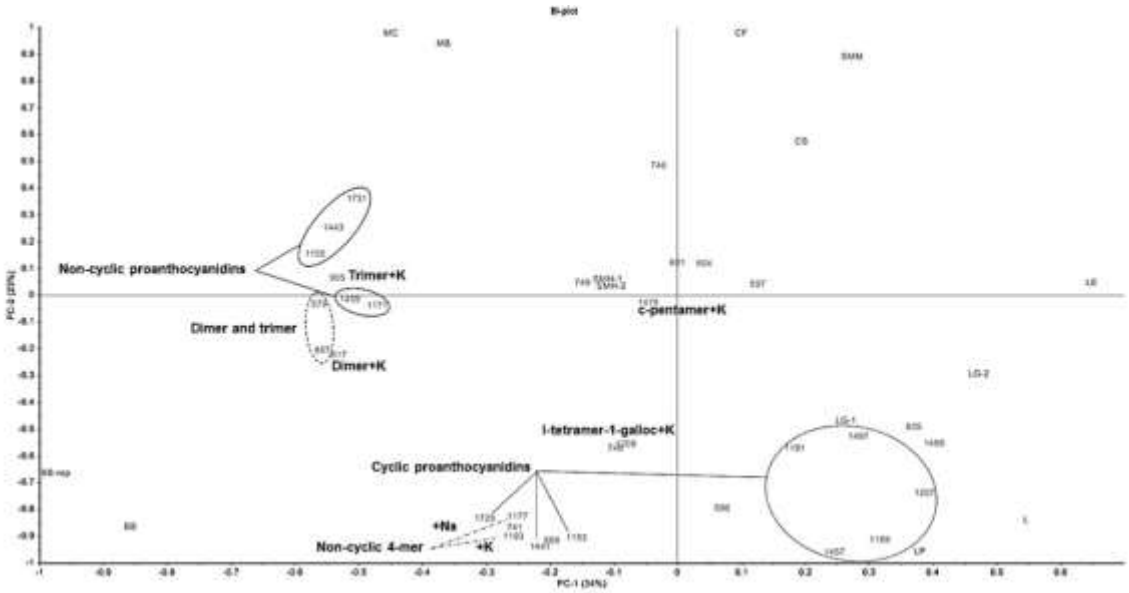


**Figure 36.** Principal component analysis (PC1 vs PC2) of red wine samples. Legend: 579 = dimer; 617 = dimer-$K^+$; 601 = dimer-$Na^+$; 867 = trimer; 905 = trimer-$K^+$; 889 = trimer-$Na^+$; 1155 = l-tetramer; 1193 = l-tetramer-$K^+$; 597 = l-tetramer-$Ca^{2+}$; 1177 = l-tetramer-$Na^+$; 1153 = c-tetramer; 1191 = c-tetramer-$K^+$; 596 = c-tetramer-$Ca^{2+}$; 1443 = l-pentamer; 1481 = l-pentamer-$K^+$; 741 = l-pentamer-$Ca^{2+}$; 1441 = c-pentamer; 1479 = c-pentamer-$K^+$; 740 = c-pentamer-$Ca^{2+}$; 1731 = l-hexamer; 1769 = l-hexamer-$K^+$; 885 = l-hexamer-$Ca^{2+}$; 1729 = c-hexamer; 1767 = c-hexamer-$K^+$; 884 = c-hexamer-$Ca^{2+}$; 1171 = l-tetramer-l-galloc; 1209 = l-tetramer-l-galloc-$K^+$; 605 = l-tetramer-l-galloc- $Ca^{2+}$; 1169 = c-tetramer-l-galloc; 1207 = c-tetramer-l-galloc-$K^+$; 604 = c-tetramer-l-galloc-$Ca^{2+}$; 1459 = l-pentamer-l-galloc; 1497 = l-pentamer-l-galloc-$K^+$; 749 = l-pentamer-l-galloc-$Ca^{2+}$; 1457 = c-pentamer-l-galloc; 1495 = c-pentamer-l-galloc-$K^+$; 748 = c-pentamer-l-galloc-$Ca^{2+}$.

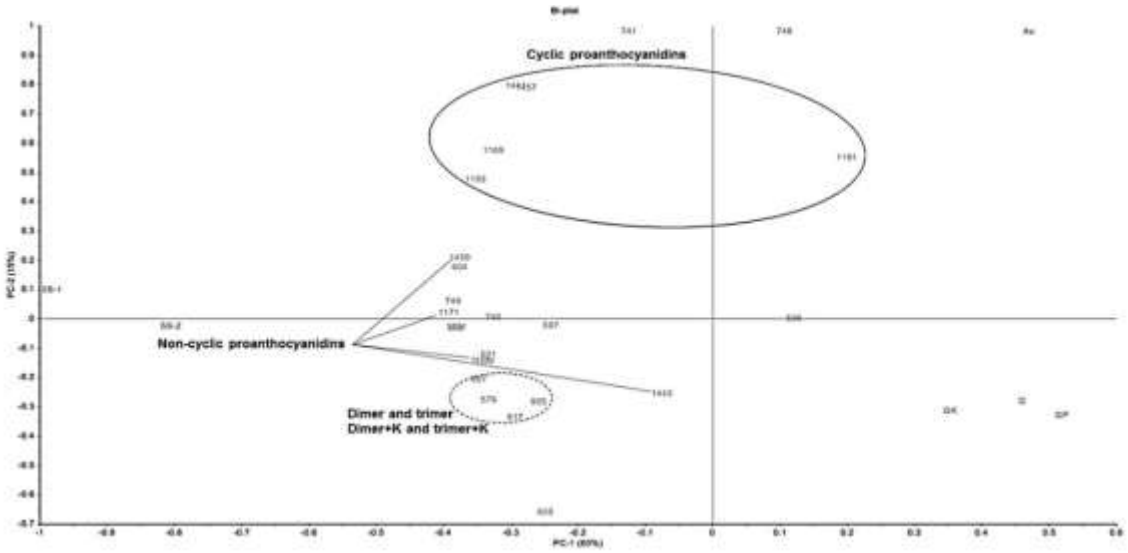


**Figure 37.** Principal component analysis (PC1 vs PC2) of white wine samples. Legend: 579 = dimer; 617 = dimer-$K^+$; 601 = dimer-$Na^+$; 867 = trimer; 905 = trimer-$K^+$; 889 = trimer-$Na^+$; 1155 = l-tetramer; 1193 = l-tetramer-$K^+$; 597 = l-tetramer-$Ca^{2+}$; 1177 = l-tetramer-$Na^+$; 1153 = c-tetramer; 1191 = c-tetramer-$K^+$; 596 = c-tetramer-$Ca^{2+}$; 1443 = l-pentamer; 1481 = l-pentamer-$K^+$; 741 = l-pentamer-$Ca^{2+}$; 1441 = c-pentamer; 1479 = c-pentamer-$K^+$; 740 = c-pentamer-$Ca^{2+}$; 1731 = l-hexamer; 1769 = l-hexamer-$K^+$; 885 = l-hexamer-$Ca^{2+}$; 1729 = c-hexamer; 1767 = c-hexamer-$K^+$; 884 = c-hexamer-$Ca^{2+}$; 1171 = l-tetramer-l-galloc; 1209 = l-tetramer-l-galloc-$K^+$; 605 = l-tetramer-l-galloc- $Ca^{2+}$; 1169 = c-tetramer-l-galloc; 1207 = c-tetramer-l-galloc-$K^+$; 604 = c-tetramer-l-galloc-$Ca^{2+}$; 1459 = l-pentamer-l-galloc; 1497 = l-pentamer-l-galloc-$K^+$; 749 = l-pentamer-l-galloc-$Ca^{2+}$; 1457 = c-pentamer-l-galloc; 1495 = c-pentamer-l-galloc-$K^+$; 748 = c-pentamer-l-galloc-$Ca^{2+}$.

The use of technological approaches (e.g. use of metatartaric acid or bentonite) could also have had an impact on the results. However, the sample LG-2 underwent a cold treatment with no addition of metatartaric acid and its difference from LG-1 it is not so defined. The addition of metatartaric acid does not alter the overall concentration of potassium in solution but just its rate of crystallization, therefore it is possible that the binding with PAC was not so affected as the rate of crystal formation/growth. On the contrary, cold treatment should lead to an overall loss of potassium from the wine which should have had an effect on the potassium complex amount. Again, a neat distinction between red and white wines is observed, which was not so foreseeable this time (Longo *et al.,* 2008a; 2018c) for the similar aforementioned reasons. This could suggest that the relative abundance of specific oligomers may be again the most important variable to take into account, with a less defined contribution from metal binding.

## 3.4 Conclusion

Proanthocyanidins complex with potassium and calcium metals were screened in 19 wines from the South-Tyrolean region. An HLPC/HRMS/MS approach allowed to identify several candidates and to highlight the probable contribution from specific isomeric forms at several n-meric stages. Namely, potassium appeared to bind selectively to one non-cyclic trimer (the most polar one in particular). Then, calcium and potassium appeared to bind more to the single cyclic tetramer procyanidin than to the many non-cyclic tetramer procyanidin. This is an example of how conformation (cyclic vs non-cyclic) affected the selectivity. Similarly, potassium bound preferentially to the cyclic pentameric procyanidin whereas calcium preferred the non-cyclic congener. In addition, calcium was found to bind to the cyclic pentameric proanthocyanidin containing l-gallocatechin than its non-cyclic congener.

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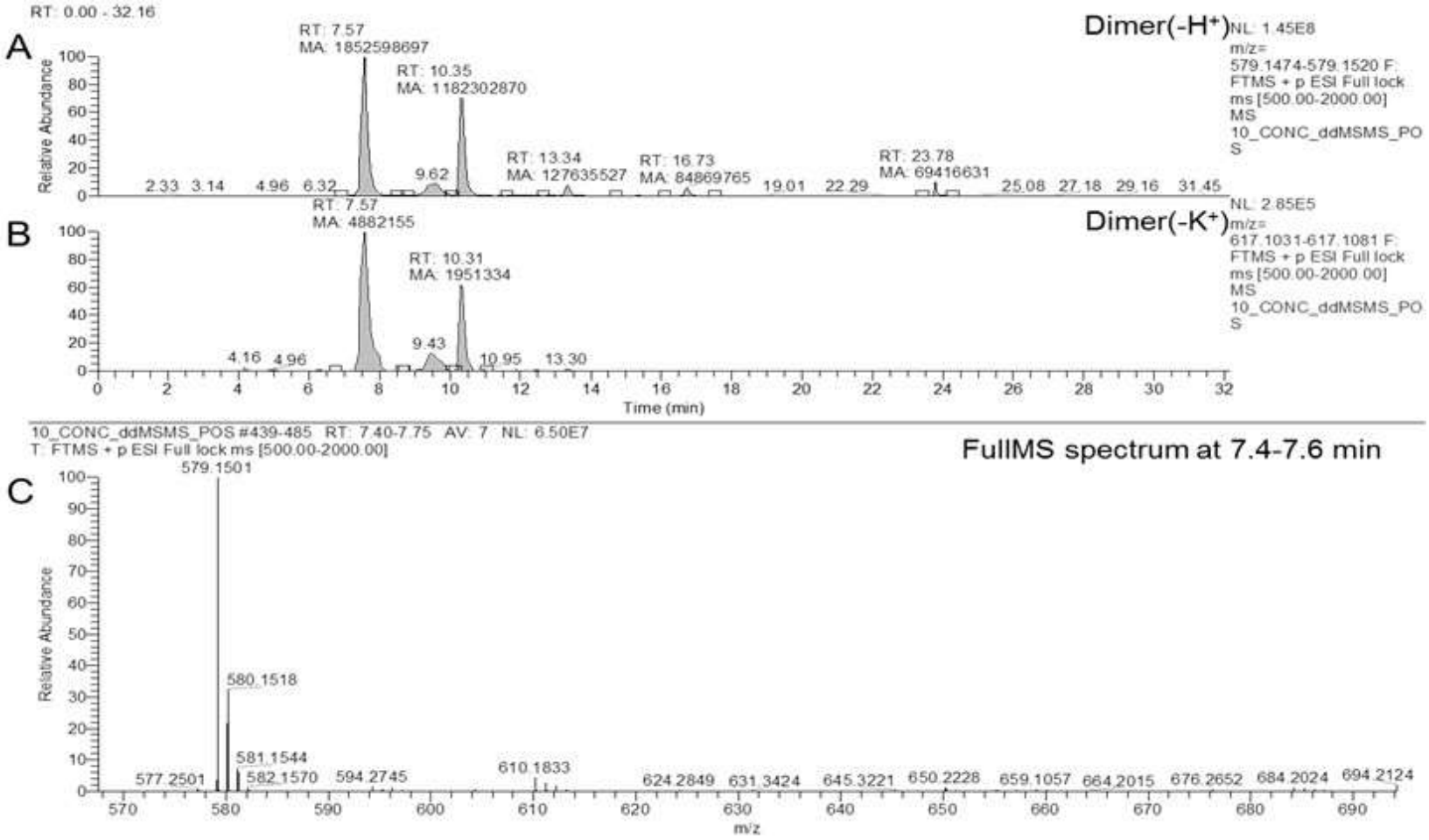


**Figure SI l**-dimer. A) EIC of [M-H]+, B) EIC of [M-K]+ and C) Full-MS spectrum at 7.4-7.6 min.

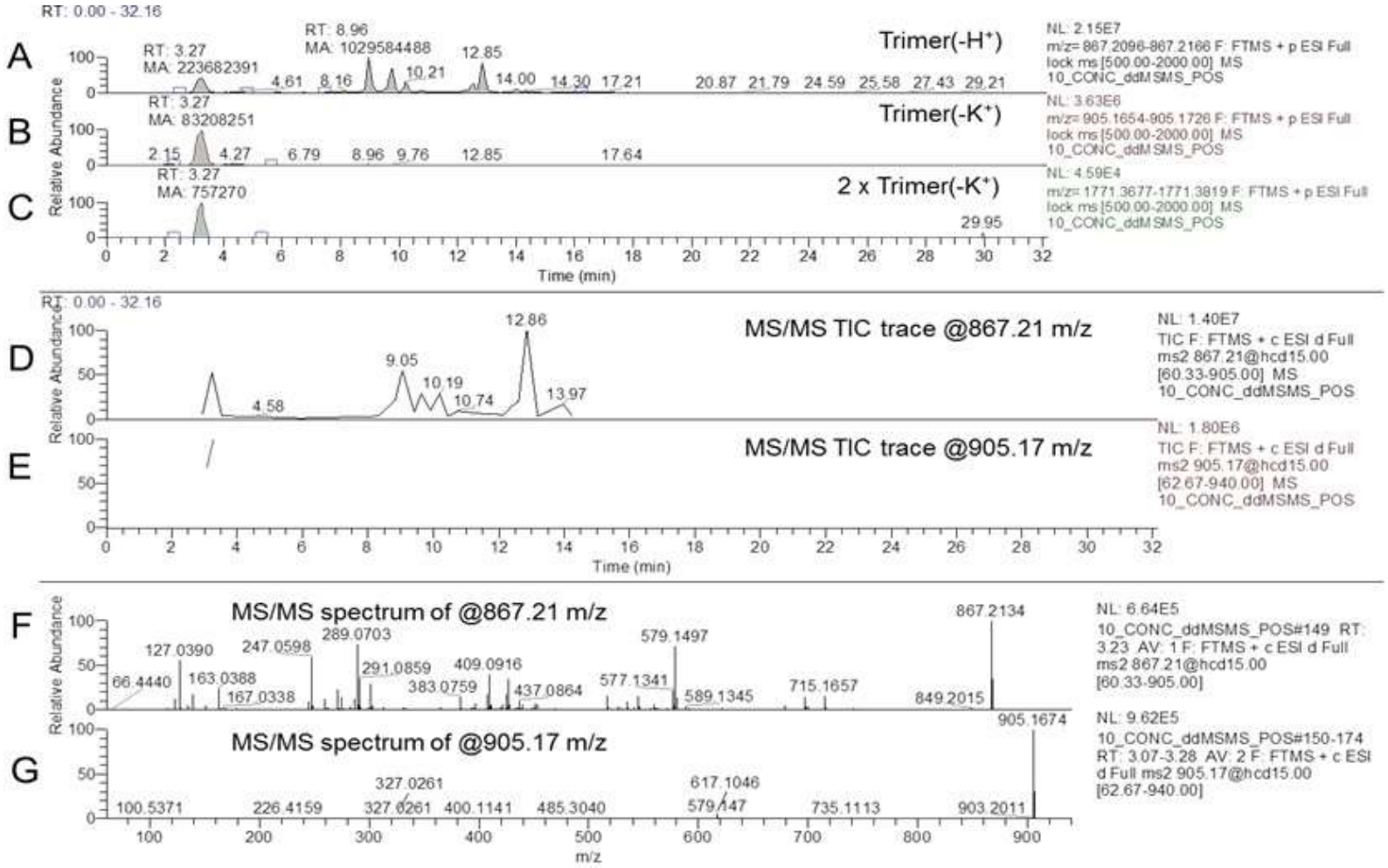


**Figure SI2** l-trimer. A) EIC of $[M-H]^+$, B) EIC of $[M-K]^+$, C) EIC of $[2M-K]^+$, D) MS/MS TIC trace for $[M-H]^+$, E) MS/MS TIC trace for $[M-K]^+$, F) MS/MS spectrum at 3.2 min for $[M-H]^+$ and G) MS/MS spectrum at 3.1 min for $[M-K]^+$.

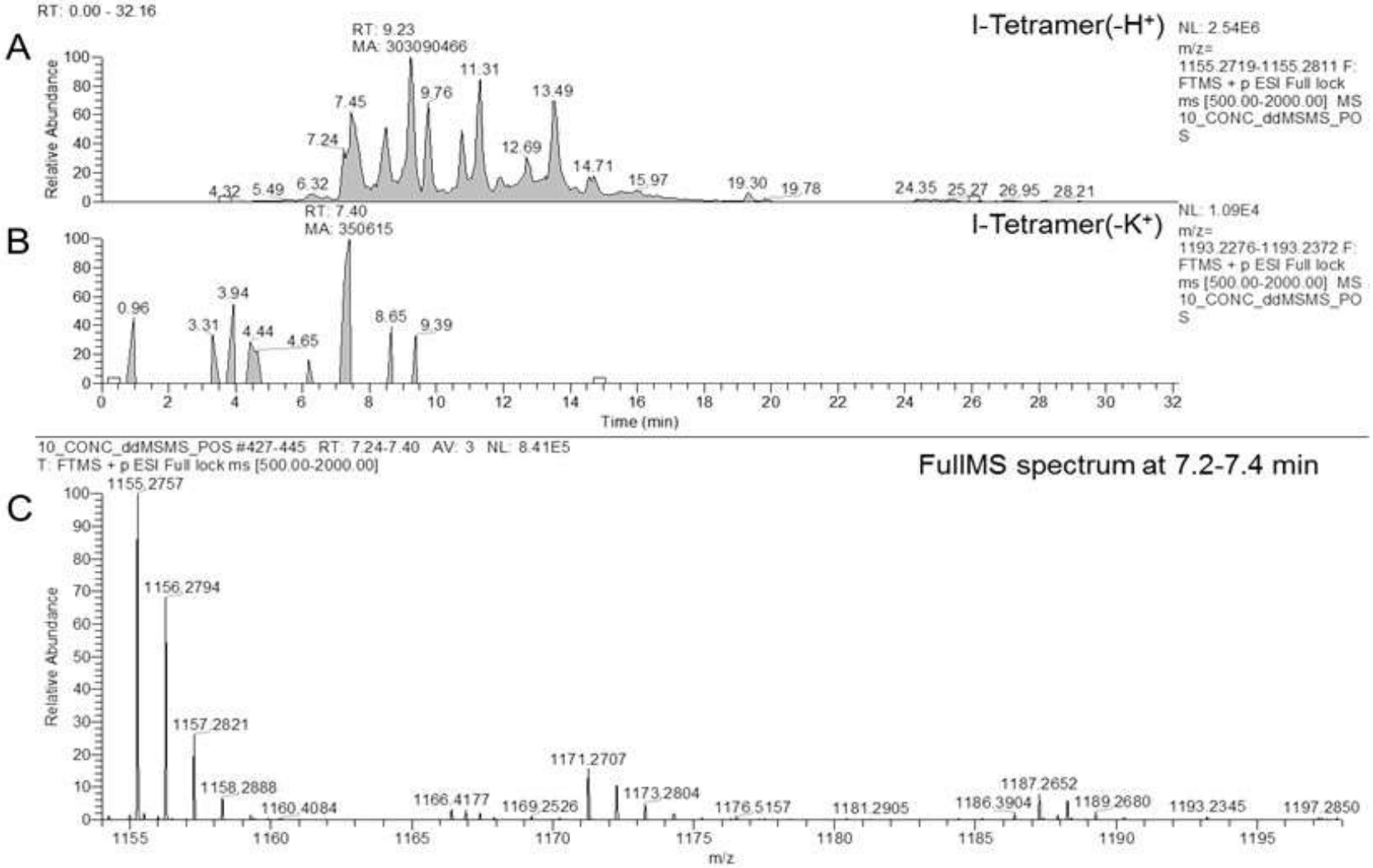


**Figure SI3** l-tetramer. A) EIC of $[M-H]^+$, B) EIC of $[M-K]^+$ and C) Full-MS spectrum at 7.2-7.4 min.

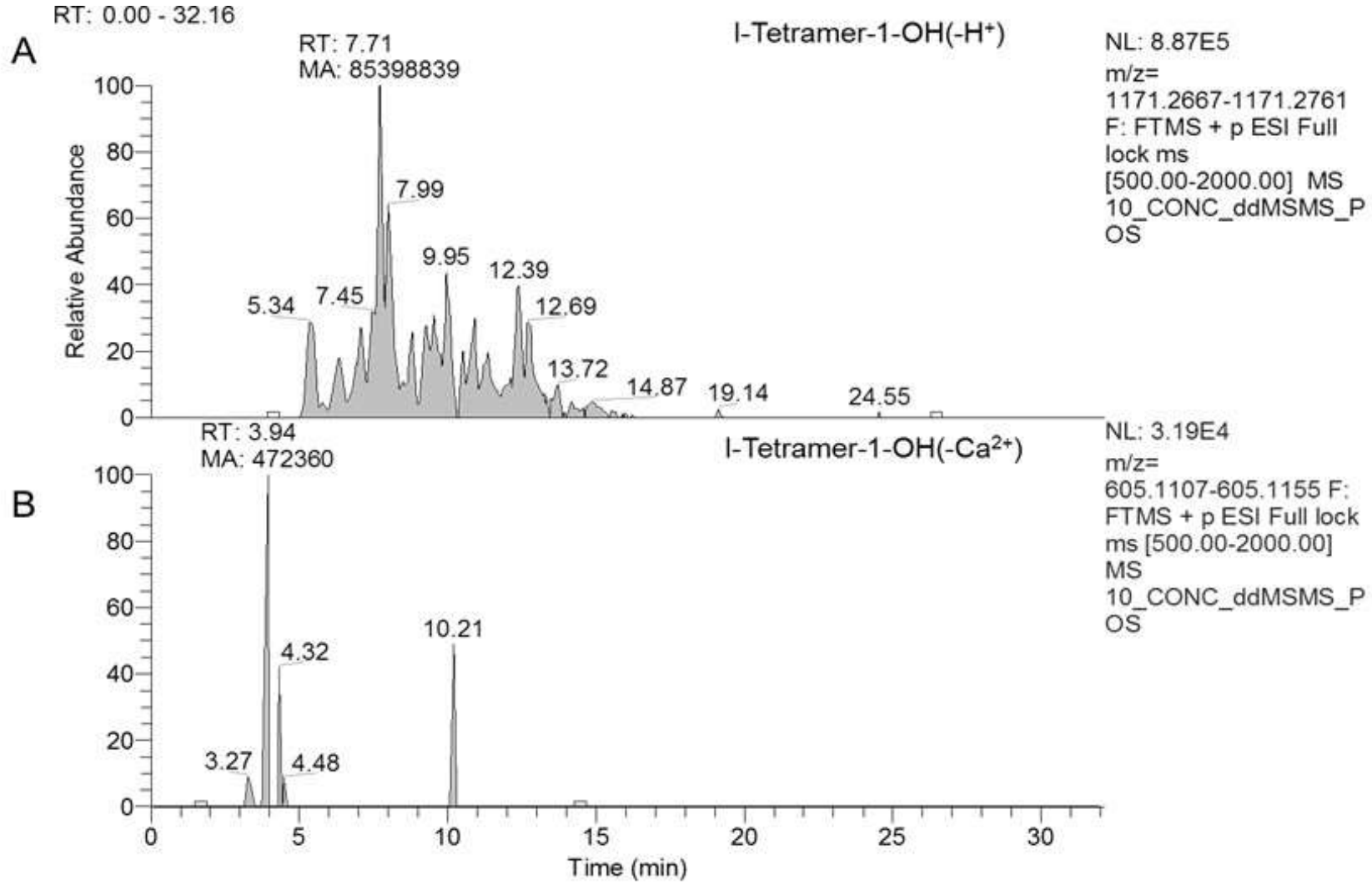


**Figure SI4** l-tetramer-l-galloc. A) EIC of $[M-H]^{+}$, B) EIC of $[M-Ca]^{2+}$.

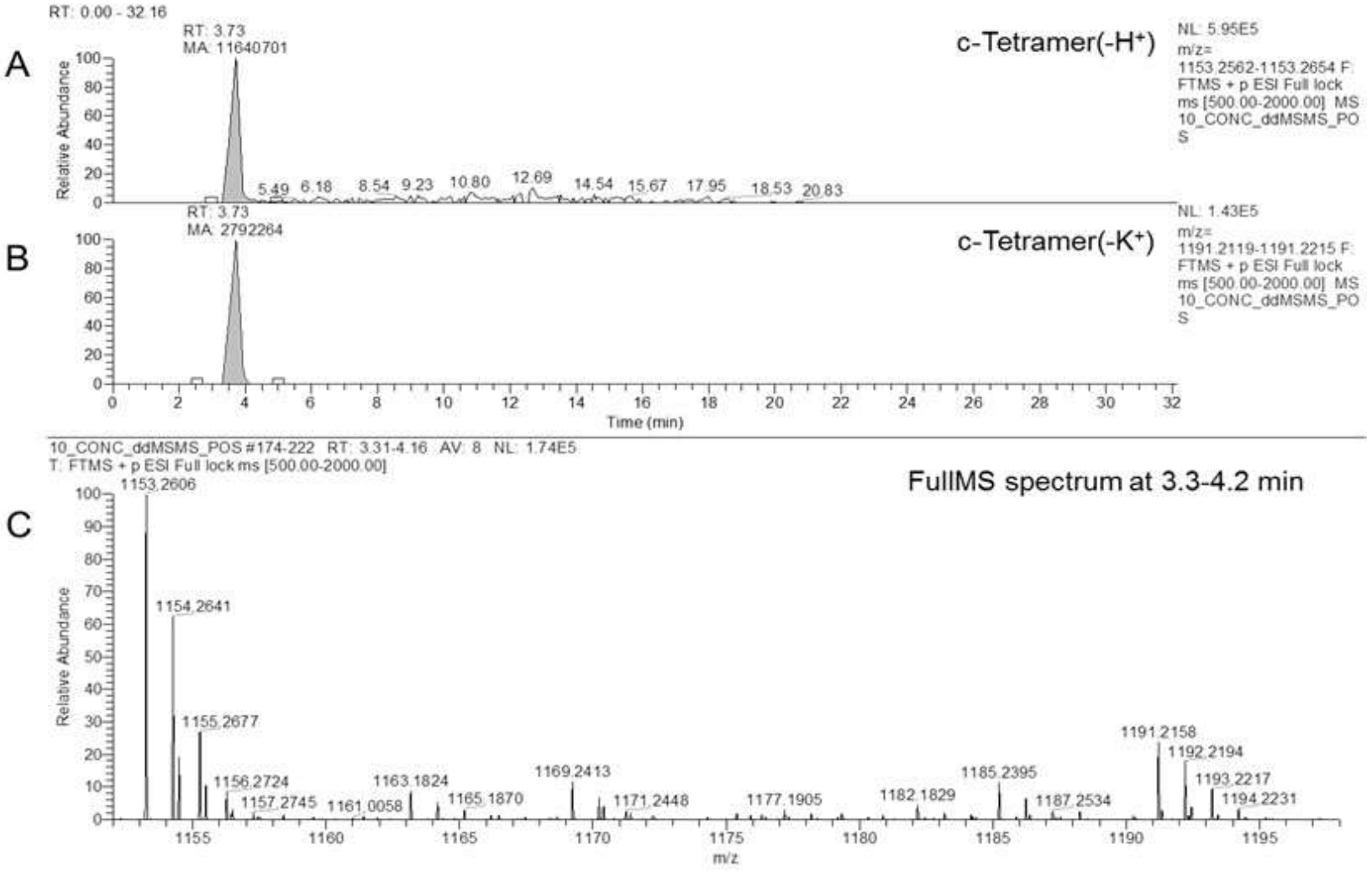


**Figure SI5** c-tetramer. A) EIC of $[M-H]^+$, B) EIC of $[M-K]^+$ and C) Full-MS spectrum at 3.3-4.2 min.

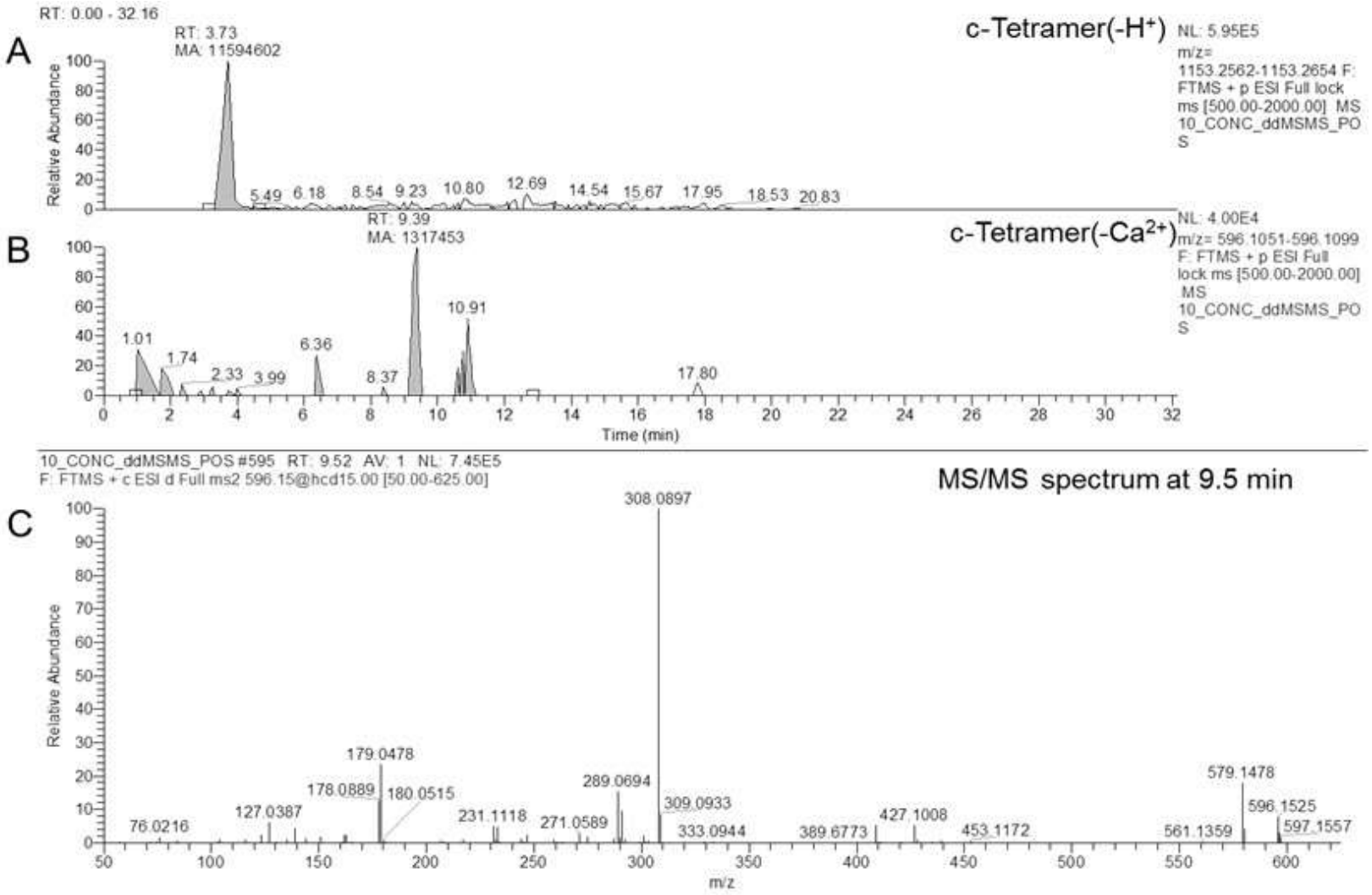


**Figure SI6** c-tetramer. A) EIC of $[M-H]^+$, B) EIC of $[M-Ca]^{2+}$ and C) MS/MS spectrum at 9.5 min.

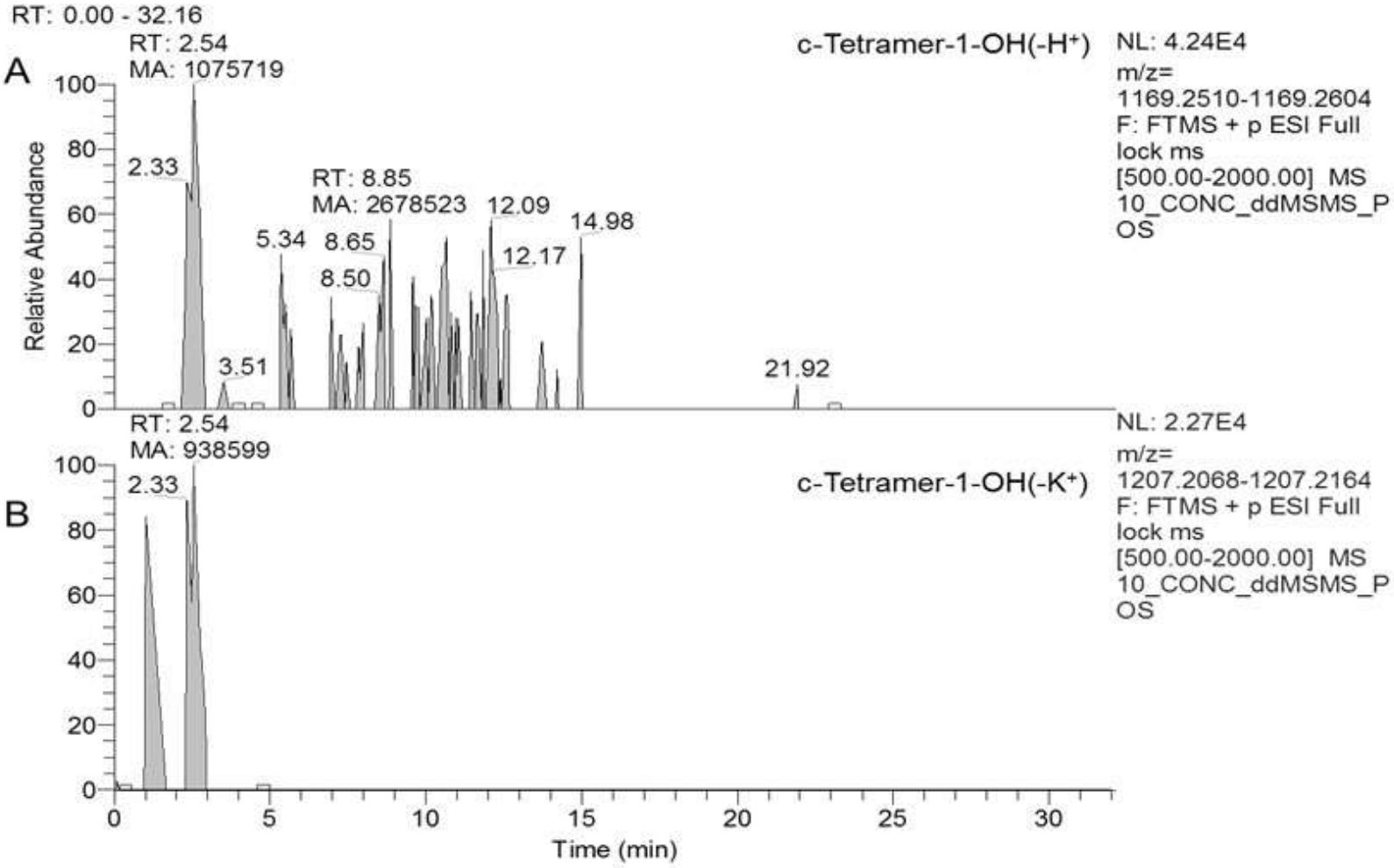


**Figure SI7** c-tetramer-1-galloc. A) EIC of $[M-H]^+$, B) EIC of $[M-K]^+$.

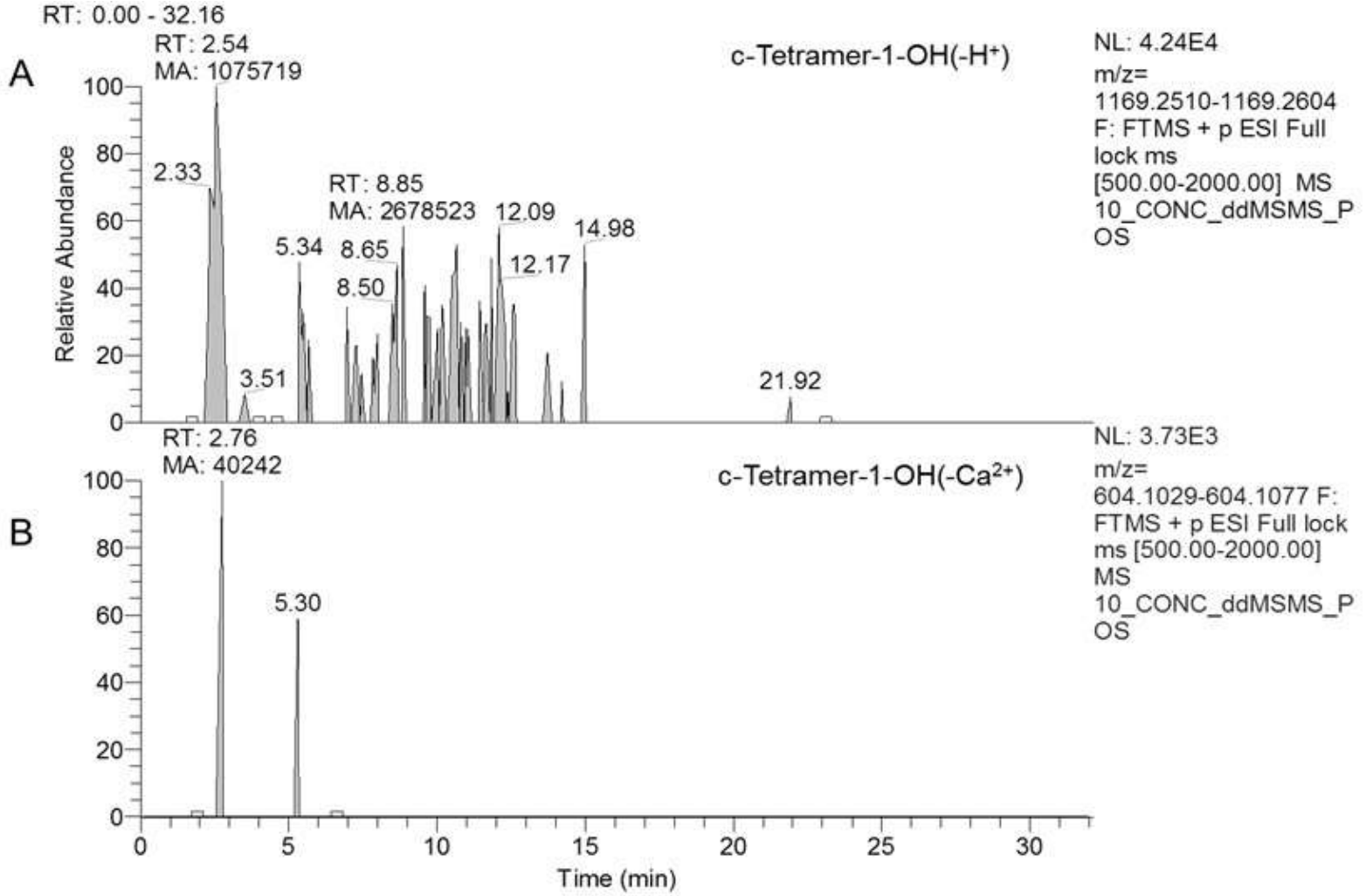


**Figure SI8** c-tetramer-l-galloc. A) EIC of $[M-H]^+$, B) EIC of $[M-Ca]^{2+}$.

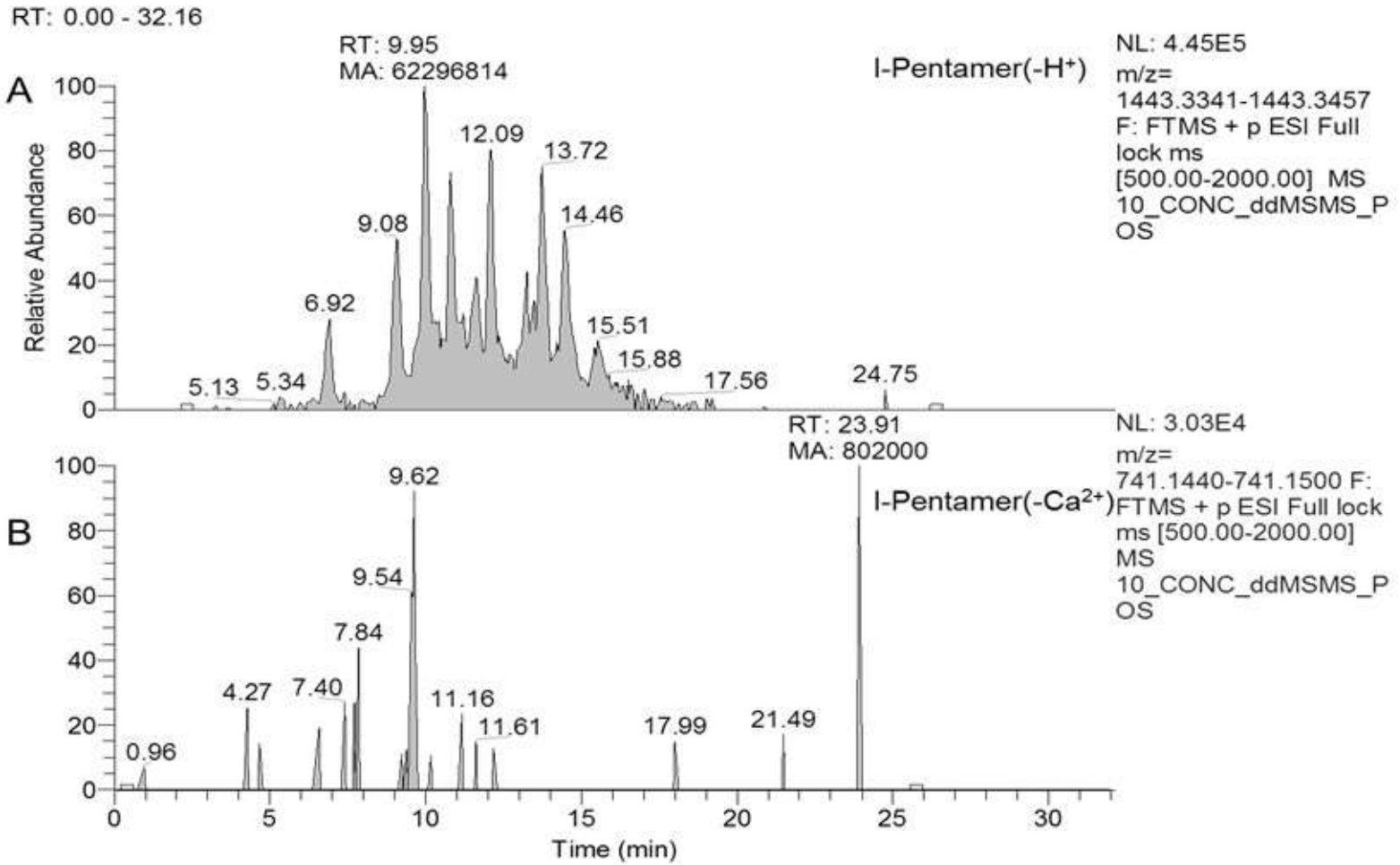


**Figure SI9** l-pentamer. A) EIC of $[M-H]^{+}$, B) EIC of $[M-Ca]^{2+}$.

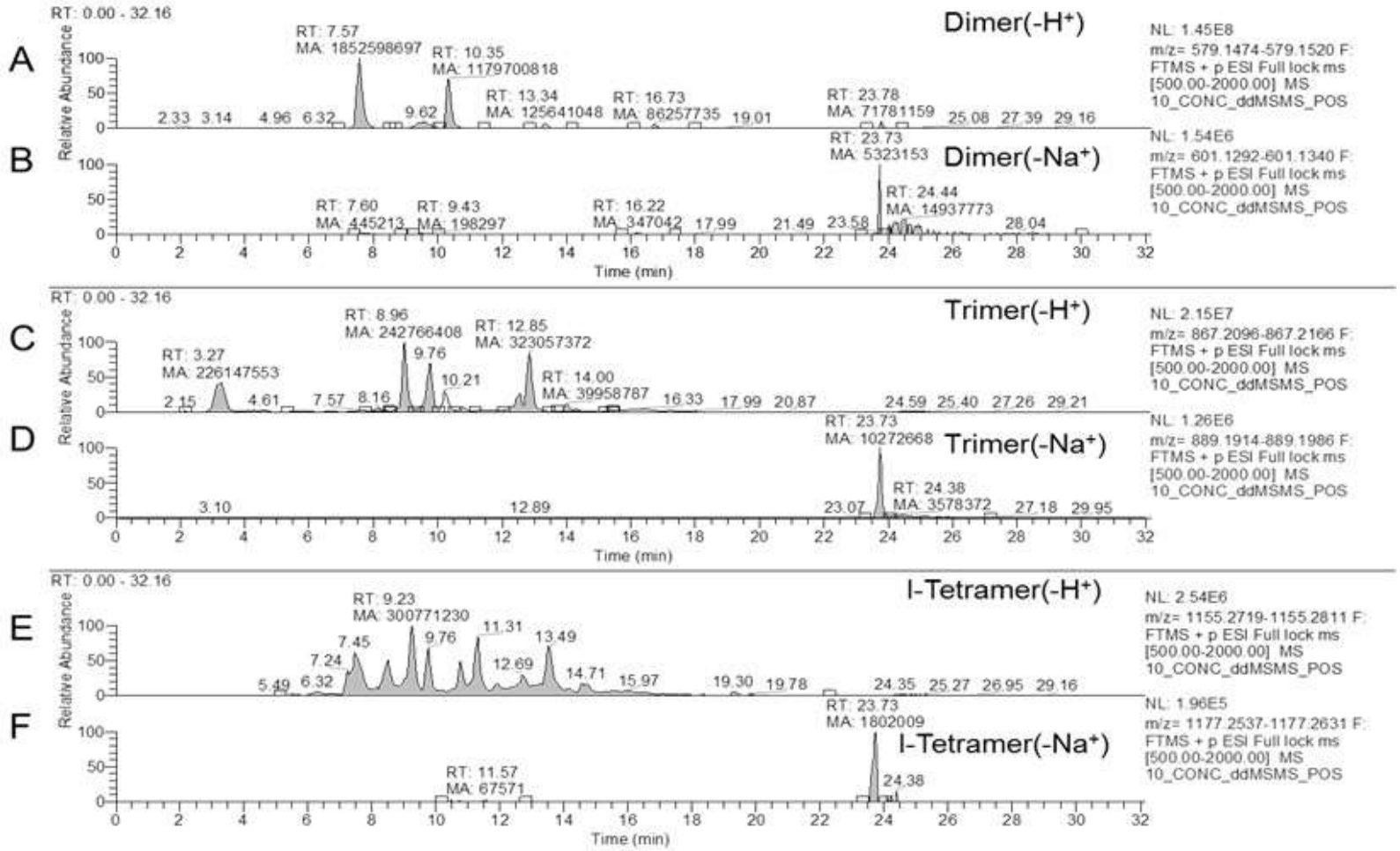


**Figure SI10** Sodium adducts EICs. A) l-dimer $[M-H]^+$, B) l-dimer $[M-Na]^+$, C) l-trimer $[M-H]^+$, D) l-trimer $[M-Na]^+$, E) l-tetramer $[M-H]^+$, F) l-tetramer $[M-Na]^+$.

**Table SI 1a** Relative abundances. Legend: 579 = dimer; 617 = dimer-$K^+$; 601 = dimer-$Na^+$; 867 = trimer; 905 = trimer-$K^+$; 889 = trimer-$Na^+$; 1155 = l-tetramer; 1193 = l-tetramer-$K^+$; 597 = l-tetramer-$Ca^{2+}$; 1177 = l-tetramer-$Na^+$.

| | VARIETY | **579** | **617** | **601** | **867** | **905** | **889** | **1155** | **1193** | **597** | **1177** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| L | Lagrein | 931626012 | 1550529 | 28864344 | 32452620 | 724435 | 1349065 | 51640388 | 50332 | 0 | 68890 |
| LP | Lagrein | 910558346 | 1369546 | 14062631 | 34792068 | 704729 | 6241134 | 48979335 | 21982 | 0 | 732345 |
| LE | Lagrein | 609076489 | 1092398 | 14952830 | 18458503 | 482578 | 2052361 | 25625378 | 3889 | 0 | 281783 |
| LG-1 | Lagrein | 926906460 | 1650215 | 8790008 | 35393545 | 787431 | 5378387 | 56348484 | 22118 | 0 | 858115 |
| LG-2 | Lagrein | 887486049 | 1667159 | 9943654 | 21289959 | 717196 | 2976379 | 46987356 | 29772 | 31664 | 429128 |
| CF | Cabernet Franc | 1342260146 | 2023800 | 21934870 | 59115534 | 1090798 | 971985 | 79064281 | 0 | 3094 | 109194 |
| CS | Cabernet Sauvignon | 901412185 | 1278891 | 20322995 | 60060862 | 1056065 | 768998 | 67809426 | 4660 | 0 | 0 |
| MC | Merlot | 2014737653 | 2218550 | 7604030 | 108180682 | 1428004 | 198029 | 142922327 | 18168 | 0 | 7255 |
| MB | Merlot | 1781004274 | 2400980 | 39024884 | 74790572 | 1342598 | 912892 | 147574351 | 10062 | 8462 | 224026 |
| BB | Pinot Noir | 2263206658 | 3107559 | 13306111 | 196557225 | 1799008 | 6781096 | 158849111 | 43588 | 0 | 959954 |
| BB-rep | Pinot Noir | 2709373117 | 4357846 | 14889518 | 192817999 | 1873754 | 7366821 | 170882101 | 58840 | 0 | 1216117 |
| SMM | StMagdalener | 846946633 | 1214908 | 349589 | 30234575 | 807389 | 64671 | 59203692 | 11089 | 0 | 0 |
| SMH-1 | StMagdalener | 1199604673 | 2721514 | 280763 | 57288483 | 1529073 | 14328 | 79208852 | 29533 | 0 | 0 |
| SMH-2 | StMagdalener | 1054189273 | 2499921 | 526699 | 64013438 | 1818990 | 11961 | 76706174 | 24515 | 0 | 4285 |
| GK | Gewürztraminer | 181422346 | 817638 | 108418 | 5877407 | 87862 | 8939 | 2063999 | 0 | 20268 | 0 |
| G | Gewürztraminer | 142639657 | 718188 | 730473 | 6204083 | 100694 | 17294 | 2076104 | 0 | 0 | 0 |
| GP | Gewürztraminer | 2873278 | 53055 | 10097916 | 190551 | 4164 | 549306 | 33086 | 0 | 0 | 84905 |
| SB-1 | Sauvignon Blanc | 293620722 | 1017290 | 21205177 | 11735041 | 109295 | 2421894 | 3762904 | 0 | 55855 | 145722 |
| SB-2 | Sauvignon Blanc | 245168849 | 1014943 | 12899108 | 10350663 | 111252 | 2108810 | 3539547 | 0 | 0 | 149432 |
| Au | Chardonnay | 2328781l | 148655 | 237705 | 1234858 | 28550 | 5285 | 714961 | 0 | 0 | 0 |

**Table SI 1b** Relative abundances. Legend: 1153 = c-tetramer; 1191 = c-tetramer-$K^+$; 596 = c-tetramer-$Ca^{2+}$; 1443 = l-pentamer; 1481 = l-pentamer-$K^+$; 741 = l-pentamer-$Ca^{2+}$; 1441 = c-pentamer; 1479 = c-pentamer-$K^+$; 740 = c-pentamer-$Ca^{2+}$.

| | VARIETY | **1153** | **1191** | **596** | **1443** | **1481** | **741** | **1441** | **1479** | **740** |
|---|---|---|---|---|---|---|---|---|---|---|
| L | Lagrein | 3541397 | 70613 | 524610 | 11913769 | 0 | 284753 | 3956111 | 0 | 0 |
| LP | Lagrein | 6079986 | 90134 | 1237745 | 10933328 | 0 | 211153 | 4667624 | 21764 | 25271 |
| LE | Lagrein | 3891604 | 49560 | 234243 | 5965993 | 0 | 103881 | 2436956 | 4388 | 24097 |
| LG-1 | Lagrein | 4922892 | 45805 | 1471366 | 12512386 | 0 | 307410 | 3429915 | 11015 | 8187 |
| LG-2 | Lagrein | 5155180 | 63382 | 956769 | 10485730 | 0 | 193372 | 2498369 | 3326 | 75819 |
| CF | Cabernet Franc | 1977528 | 19553 | 593727 | 19122549 | 0 | 161763 | 2127922 | 0 | 98303 |
| CS | Cabernet Sauvignon | 4469359 | 88943 | 189155 | 13518861 | 0 | 280867 | 2893638 | 25644 | 35930 |
| MC | Merlot | 3468012 | 15416 | 156021 | 35771730 | 0 | 32664 | 1353828 | 13725 | 15117 |
| MB | Merlot | 3066587 | 28586 | 222982 | 36027698 | 0 | 94973 | 1470529 | 3026 | 96736 |
| BB | Pinot Noir | 6426206 | 69431 | 659769 | 33673553 | 0 | 479593 | 6117796 | 7422 | 54187 |
| BB-rep | Pinot Noir | 5423980 | 20189 | 723105 | 35987969 | 0 | 429394 | 5295342 | 5468 | 5414 |
| SMM | StMagdalener | 3005398 | 28947 | 249736 | 11499640 | 0 | 135626 | 2363664 | 0 | 8402 |
| SMH-1 | StMagdalener | 5993076 | 107318 | 852458 | 18712919 | 0 | 243910 | 4937768 | 39890 | 0 |
| SMH-2 | StMagdalener | 5342649 | 45719 | 593455 | 14003414 | 0 | 242286 | 5082485 | 52819 | 0 |
| GK | Gewürztraminer | 233579 | 0 | 43126 | 0 | 0 | 12244 | 73284 | 0 | 9886 |
| G | Gewürztraminer | 288882 | 9247 | 166512 | 7518 | 0 | 10870 | 23576 | 0 | 6661 |
| GP | Gewürztraminer | 28774 | 0 | 16753 | 28581 | 0 | 29855 | 0 | 0 | 12512 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SB-1 | Sauvignon Blanc | 1008813 | 0 | 58039 | 17649 | 0 | 47504 | 375005 | 0 | 131036 |
| SB-2 | Sauvignon Blanc | 1071742 | 0 | 16343 | 12564 | 0 | 37795 | 320584 | 0 | 33461 |
| Au | Chardonnay | 604755 | 8213 | 65800 | 5704 | 0 | 64093 | 319709 | 0 | 0 |

**Table SI 1c** Relative abundances. Legend: 1731 = l-hexamer; 1769 = l-hexamer-$K^+$; 885 = l-hexamer-$Ca^{2+}$; 1729 = c-hexamer; 1767 = c-hexamer-$K^+$; 884 = c-hexamer-$Ca^{2+}$; 1171 = l-tetramer-1-galloc; 1209 = l-tetramer-1-galloc-$K^+$; 605 = l-tetramer-1-galloc- $Ca^{2+}$.

| | VARIETY | **1731** | **1769** | **885** | **1729** | **1767** | **884** | **1171** | **1209** | **605** |
|---|---|---|---|---|---|---|---|---|---|---|
| L | Lagrein | 2037202 | 0 | 0 | 314810 | 0 | 0 | 31645388 | 3212 | 1317348 |
| LP | Lagrein | 2006987 | 0 | 0 | 320282 | 0 | 0 | 32151954 | 5197 | 1090490 |
| LE | Lagrein | 827755 | 0 | 0 | 272017 | 0 | 0 | 26564787 | 0 | 1351747 |
| LG-1 | Lagrein | 2225489 | 0 | 0 | 245010 | 0 | 0 | 29565779 | 0 | 145101 |
| LG-2 | Lagrein | 1869115 | 0 | 0 | 244908 | 0 | 0 | 25908453 | 3976 | 316794 |
| CF | Cabernet Franc | 3679339 | 0 | 0 | 110498 | 0 | 0 | 25933924 | 0 | 240090 |
| CS | Cabernet Sauvignon | 2265299 | 0 | 0 | 158825 | 0 | 0 | 29231901 | 0 | 263960 |
| MC | Merlot | 7744947 | 0 | 0 | 105321 | 0 | 0 | 52992023 | 0 | 79493 |
| MB | Merlot | 8055176 | 0 | 0 | 117407 | 0 | 0 | 43900776 | 0 | 161899 |
| BB | Pinot Noir | 5952118 | 0 | 0 | 684666 | 0 | 0 | 46867234 | 9296 | 135739 |
| BB-rep | Pinot Noir | 6662481 | 0 | 0 | 610440 | 0 | 0 | 48406246 | 0 | 96552 |
| SMM | StMagdalener | 1788047 | 0 | 0 | 298725 | 0 | 0 | 22057695 | 0 | 85883 |
| SMH-1 | StMagdalener | 3399338 | 0 | 0 | 439304 | 0 | 0 | 26637411 | 0 | 216265 |
| SMH-2 | StMagdalener | 2544180 | 0 | 0 | 387137 | 0 | 0 | 27725379 | 11822 | 147141 |
| GK | Gewürztraminer | 0 | 0 | 0 | 0 | 0 | 0 | 76919 | 0 | 57895 |
| G | Gewürztraminer | 0 | 0 | 0 | 0 | 0 | 0 | 61442 | 0 | 73785 |
| GP | Gewürztraminer | 2429 | 0 | 0 | 0 | 0 | 0 | 2400 | 0 | 66967 |
| SB-1 | Sauvignon Blanc | 10977 | 0 | 0 | 0 | 0 | 0 | 737574 | 0 | 73292 |
| SB-2 | Sauvignon Blanc | 9350 | 0 | 0 | 0 | 0 | 0 | 578333 | 0 | 106500 |
| Au | Chardonnay | 0 | 0 | 0 | 0 | 0 | 0 | 21176 | 0 | 27927 |

**Table SI 1d** Relative abundances. Legend: 1169 = c-tetramer-1-galloc; 1207 = c-tetramer-1-galloc-$K^+$; 604 = c-tetramer-1-galloc-$Ca^{2+}$; 1459 = l-pentamer-1-galloc; 1497 = l-pentamer-1-galloc-$K^+$; 749 = l-pentamer-1-galloc-$Ca^{2+}$; 1457 = c-pentamer-1-galloc; 1495 = c-pentamer-1-galloc-$K^+$; 748 = c-pentamer-1-galloc-$Ca^{2+}$.

| | VARIETY | **1169** | **1207** | **604** | **1459** | **1497** | **749** | **1457** | **1495** | **748** |
|---|---|---|---|---|---|---|---|---|---|---|
| L | Lagrein | 5170014 | 154114 | 84914 | 6929081 | 10114 | 0 | 4782762 | 46567 | 5669271 |
| LP | Lagrein | 4724990 | 111830 | 35483 | 6368516 | 5552 | 0 | 4346304 | 11058 | 4738489 |
| LE | Lagrein | 3125234 | 78473 | 33336 | 5634775 | 0 | 5357 | 4568092 | 31436 | 3022903 |
| LG-1 | Lagrein | 3638473 | 105955 | 41963 | 6498645 | 0 | 0 | 2943117 | 22038 | 2501831 |
| LG-2 | Lagrein | 3081070 | 68802 | 59262 | 5781177 | 12712 | 13741 | 3042337 | 28410 | 2769267 |
| CF | Cabernet Franc | 756343 | 0 | 104314 | 5580363 | 0 | 21617 | 671575 | 0 | 932228 |
| CS | Cabernet Sauvignon | 794944 | 0 | 159083 | 6374028 | 0 | 0 | 1383017 | 3511 | 1841861 |
| MC | Merlot | 348787 | 0 | 22530 | 13474409 | 0 | 0 | 893307 | 0 | 1699769 |
| MB | Merlot | 733882 | 0 | 13318 | 11107382 | 0 | 18060 | 838676 | 0 | 6640851 |
| BB | Pinot Noir | 1561203 | 0 | 119103 | 12360868 | 0 | 38133 | 2481632 | 0 | 5863179 |
| BB-rep | Pinot Noir | 1602966 | 0 | 25104 | 13517050 | 0 | 29720 | 2662360 | 0 | 3071580 |
| SMM | StMagdalener | 453465 | 0 | 91214 | 5069834 | 0 | 25862 | 535421 | 0 | 364498 |
| SMH-1 | StMagdalener | 911895 | 0 | 160322 | 6712404 | 0 | 160535 | 1022748 | 0 | 908586 |
| SMH-2 | StMagdalener | 831610 | 21797 | 38861 | 6318043 | 0 | 106904 | 1424783 | 2379 | 1832696 |
| GK | Gewürztraminer | 18284 | 0 | 32448 | 0 | 0 | 0 | 0 | 0 | 15451 |
| G | Gewürztraminer | 1862 | 0 | 30720 | 0 | 0 | 0 | 3401 | 0 | 15000 |
| GP | Gewürztraminer | 0 | 0 | 24051 | 0 | 0 | 0 | 0 | 0 | 0 |
| SB-1 | Sauvignon Blanc | 111735 | 0 | 127441 | 22769 | 0 | 391877 | 98206 | 0 | 9191 |
| SB-2 | Sauvignon Blanc | 149926 | 0 | 158429 | 28709 | 0 | 444651 | 50124 | 0 | 10896 |
| Au | Chardonnay | 85445 | 0 | 48443 | 5297 | 0 | 13105 | 69421 | 0 | 37301 |

# General Conclusion

Traditions are strongly rooted in the world of enology because it ensures authenticity of the product and fascinates the consumer. However, the application of technologies is essential for contemporary wine producers. Nowadays, the revisiting of the past methodologies is often combined with the application of modern technologies with the aim of obtaining controlled products without defects and with a stable quality.
The aim of this thesis was the investigation of three methodologies nowadays widespread between winemakers.

**Chapter I**

The first outcome was that the nature of the container resulted to be a potential factor differentiating chemical and sensory parameters of wines obtained from the same Chardonnay grapes. By using the modern amphorae, winemakers may extend their commercial wine offer by exalting the characteristics of the grape in a different and innovative way, not related to the ageing in wood.
Moreover, insights on the chemical properties of a modern in-amphorae wine showed that it was characterized by a higher content of free phenolic acids (catechin, caffeic acid, *p*-coumaric acid, epicatechin and protocatechuic acid) and of higher volatile alcohols compared with the wooden containers. The sensory evaluation differentiated in-amphorae wines compared with the other containers through four attributes: solvent and acetone, astringent/pungency, fruity, and color intensity.

**Chapter II**

The impact of the Supercap Nature stopper was evaluated on four Italian wines from South Tyrol – Alto Adige in comparison with conventional cork-based stoppers. Statistic results did not report a constant trend of significant differences between the wines closed with the two stoppers. The reduction of the phenolic compounds and the evolution of the volatiles resulted foremost influenced by non-oxygen mediated reactions occurring during the storage in bottle. The triangle test showed differences for the Merlot wine samples stored for six months. The PCA of the volatile compounds allowed to speculate about the influence of the ethyl dodecanoate (and its

interactions) on the sensory profile of the Merlot sample closed with the conventional stopper. However, this was the only case where the assessors correctly recognized the odd sample. The investigation on the type of stopper resulted in the similar influence of the Supercap Nature stopper compared to the conventional one during twelve months of storage in bottle.

**Chapter III**

Recent investigation on wine proanthocyanidins discovered a novel class with cyclic structure named crown tannins. In this work the high-performance liquid chromatography/high resolution tandem mass spectrometry (HPLC/HRMS/MS) method was applied to study the binding of cyclic and non-cyclic PACs to potassium and calcium ions in 19 wines from the South-Tyrolean region. The results showed that potassium bound preferentially to the cyclic pentameric procyanidin whereas calcium preferred the non-cyclic congener. This qualitative investigation would provide preliminary results for considering cyclic proanthocyanidins as new chemical markers of wine quality and authenticity. The wide variety of these species allowed the proposal that their relative abundances could be used as tools for differentiating the wines by grape variety.

# Annex I / Research Products

**Publications in Journals with Impact Factor**

Rossetti F, Boselli E (2017) Effects of in amphorae winemaking on the chemical and sensory profile of Chardonnay wine. Scientia Agriculturae Bohemica 48: 39-46. 10.1515/sab-2017-0006

Rossetti F, Merkyte V, Longo E, Pavlic B, Jourdes M, Teissedre PL, Boselli E (2018) Volatile, phenolic and sensory profile of in-amphorae Chardonnay wine by mass spectrometry and chemometric analysis. J Mass Spectrom 53(9): 833-841. https://doi.org/10.1002/jms.4262

Longo E, Rossetti F, Merkyte V, Boselli E (2018) Disambiguation of Isomeric Procyanidins with Cyclic B-Type and Non-cyclic A-Type Structures from Wine and Peanut Skin with HPLC-HDX-HRMS/MS J. Am. Soc. Mass Spectrom. 29(11) 2268-2277. 10.1007/s13361-018-2044-5

Longo E, Rossetti F, Merkyte V, Obiedzińska A, Boselli E (2018) Selective binding of potassium and calcium ions to novel cyclic proanthocyanidins in wine by HPLC-HRMS. Rapid Commun Mass Spectrom. 32(18): 1637-1642. https://doi.org/10.1002/rcm.8221

Longo E, Vakare M, Rossetti F, Teissedre PL, Jourdes M, Boselli E (2018) Relative abundances of novel cyclic prodelphinidins in wine depending on the grape variety. Journal of Mass Spectrometry 53(11): 1116-1125. doi.org/10.1002/jms.4280

Longo E, Rossetti F, Scampicchio M, Boselli E (2018) Isotopic exchange HPLC-HRMS/MS for the structural characterization of unconventional cyclic proanthocyanidins in wine and cranberries. J. Am. Soc. Mass Spectrom. 29(4): 663-674. 10.1007/s13361-017-1876-8

Yasar S, Boselli E, Rossetti F, Gok M.S. (2018) Effect of Fermented Cereals, Probiotics, and Phytase on the Sensory Quality of Poultry Meat. 49(3): 225–235. doi: 10.2478/sab-2018-0029

**Proceeding at congress**

Rossetti Fabrizio, Boselli Emanuele (2016) Technological innovation applied to the winemaking tradition. In XXI Workshop on the Developments. Paper presented at: *The Italian PhD research on Food Science, Technology and Biotechnology.*

University of Naples-Department of Agricultural Sciences. p. 70-71. ISBN: 9788899648060.

Jouin Alicia, Rossetti Fabrizio, Teissèdre Pierre-Louis, Jourdes Michael (2017) Evaluation of crown procyanidins contents in different variety and their accumulation kinetic during grape maturation. Paper presented at: *10th In Vino Analytica Scientia Symposium, IVAS* University of Salamanca. p. 160. ISBN: 9788469745793

Rossetti Fabrizio, Boselli Emanuele (2017) Effect of in-amphorae winemaking on the chemical and sensory profile of Chardonnay wine. Paper presented at: *XXII Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology*. Libera Università di Bolzano. p. 167-168. ISBN: 9788898416974

Rossetti Fabrizio, Jourdes Michael, Teissedre Pierre-Louis, Longo Edoardo, Boselli Emanuele (2017) UHPLC-HRMS phenolic characterization of Chardonnay wine obtained with vinification in amphorae, barrels and barrique. Paper presented at: *5th MS Food Day, Bologna.* Divisione di Spettrometria di Massa.

Rossetti Fabrizio, Boselli Emanuele (2018) Technological innovation applied to the winemaking tradition: influence of an innovative composite cork stopper on the chemical parameters of different types of wine. Paper presented at: *XXIII Workshop on the development in the Italian PhD research on food science, technology and biotechnology*. p. 324-328. ISBN 978-88-907678-6-9

Longo Edoardo, Rossetti Fabrizio, Scampicchio Matteo, Boselli Emanuele (2018) Isotopic exchange HPLC-HRMS/MS applied to cyclic proanthocyanidins in wine and cranberries. Paper presented at: *Food and Applied Bioscience International Conference*. Chiang Mai University. p. 9 Book of abstract.

Merkyte Vakare, Longo Edoardo, Rossetti Fabrizio, Boselli Emanuele (2018) Hydrogen/Deuterium exchange is an effective method for the structural resolution of isomeric proanthocyanidins in wine and other dietary sources. Paper presented at: *XXII Mass spectrometry Conference.* Società Chimica Italiana Divisione di Spettrometria di Massa. p. 1215. Book of abstract.

Boselli Emanuele, Longo Edoardo, Rossetti Fabrizio, Merkyte Vakare (2018) Novel natural markers of wine quality. Paper presented at: *The 17th International Symposium prospects for 3rd Millennium Agriculture*. University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca. p.58. ISNN 2392-6937

Rossetti Fabrizio, Merkyte Vakare, Longo Edoardo, Jouin Alicia, Jourdes Michael, Teissedre P-Louis, Boselli Emanuele (2018) Effect of different types of stopper on the phenolic, volatile and sensory characteristics of four wines from South Tyrol (Italy). Paper presented at: *International congress on Grapewine and Wine Sciences*. Instituto de Ciencias de la Vid y del Vino, Logroño, Spain. Book of Abstracts p.193.

Merkyte Vakare, Longo Edoardo, Rossetti Fabrizio, Jouin Alicia, Miramont Clemente, Jourdes Michael, Teissedre P-Louis, Boselli Emanuele (2018) Application of crown/cyclic proanthocyanidins relative abundances to assess wine authenticity. Paper presented at: *International congress on Grapewine and Wine Sciences*. Instituto de Ciencias de la Vid y del Vino, Logroño, Spain. Book of Abstracts p.187.

**Other contributions**

F. Rossetti, M. Mozzon, S. Savini, E. Boselli, J.H. Thorngate (2017) Il carattere erbaceo dei vini. Industrie delle bevande. Chirotti editori. p. 5-18. ISSN 0390-0541

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