U N I V E R S I T À D E G L I S T U D I D I M I L A N O

F A C O L T À D I A G R A R I A

Scuola di dottorato in Scienze molecolari e biotecnologie agrarie, alimentari ed ambientali

Corso di dottorato di ricerca in Biologia vegetale e produttività della pianta coltivata

(Cycle: XXVIII)

Genotype x environment interaction in grapevines: proanthocyanidins accumulations and polymerization in different biotypes of two Sicilian cultivars

Supervisor:

Prof. Osvaldo Failla

Co-supervisor:

Prof. Rocco Di Stefano

A C A D E M I C Y E A R - 2 0 1 4 – 2 0 1 5

1. INTRODUCTION

1.1 - SICILIAN VITICULTURE

For more than 2500 years Sicily has been a relevant centre of viticulture in the Mediterranean. The classic Mediterranean climate is characterized by hot summer and moderate rainfall; it is particularly suitable for what of grapevines needs. Moreover, Pantelleria is a very hot island (with Winkler Index of 2400), Erice and Etna are cooler mountain (Winkler Index of 1200 and 1800 respectively).

Sicily has 112.484 hectares of vine growing and it is the third region in the world for surface cultivated of vine after California and Bordeaux. 67% of it are white grape variety and 33% are black grape.

In the last century, there was a decline with regards to the quality of the wines due to encourage government-driven schemes pushing for higher productivity on the island. Higher yields caused an over-production of lower-quality wines, even if reduced consumer confidence, with lower revenues and a reputation tarnished. Luckily, the dramatic decrease in wine consumption since the 1980s, and the more quality-focused attitudes of wine producers, has led to excellent quality of wine Sicily's in the present day. In the last decade there has been a structural improvement in the productive sector; in fact Sicily's wines came only from traditional goblet-trained vines in the past, while nowadays come mostly from the espalier system (79%), Bush vine (11%) and trellis system (10%).

Today the plant density is 2,0 - 2,70 x 0,70 - 1,50 m and the irrigation surface is more than 60% of total surface. 140 Ru, 1103 P, 779 P, 775 P, 110 R are rootstocks, more used in this region.

Moreover, wine production is 7 mil hl every year (in Italy 45 mil hl). Sicily has 7 geographical indications (IGT), 23 denomination of origin (DOC), and 1 controlled and guaranteed denomination of origin (DOCG).

The soils and the landscapes are of particular interest when it comes to studying Sicilian viticulture. Mount Etna, Europe's tallest active volcano at 3330 m, dominates the island's eastern skyline and is responsible for the mineral-rich, dark soils which characterize the Etna DOC. Vineyards are now being planted higher up on the volcanic slopes, to capitalize on the cooler air and richer soils there. 80 km south, there are Iblei

Mountains that have an important place in eastern Sicilian wine production. On their lower slopes and the coastal plains below them, the DOCs of Siracusa, Noto, Eloro and Vittoria sweep from east to west, forming a crescent which mirrors the coastline.

The limestone hills, in western Sicily, have very influential on the grape quality. In fact the western coast of the island is covered by the Marsala DOC, and within this area fall the DOCs Alcamo, Contessa Entellina, Delia Nivolelli, Erice, Menfi, Monreale, Salaparuta, Santa Margherita di Belice and Sciacca, Sambuca di Sicilia. (Report of Istituto Regionale Vini e Oli, 2012).

The developments of molecular biology has confirmed that the varieties can be the result of small and localized paradomestication phenomena, that started ages ago, influencing the genetic structure of wild population from generation to generation.

During the centuries, thanks to numerous trade activities, some varieties arrived in Sicily, so we can think they are related to the Sicilian origins (Biagini, 2011).

The existence of thousands of grape varieties in European viticulture is a witness of the essential contribution of people who domesticated the plants, guided by their culture, their myths and their daily lifestyle.

Levadoux (1956) affirms that the secret of the origins of viticulture is in the wild grape, in the analysis of the individual which are still alive in the areas of domestication. Thus the presence of ancient grape varieties, which probably have suffered a minor process of domestication, may represent the mother vines of varieties cultivated today.

There are several consideration which are necessary to do this, including the conservation and study of ancient grape varieties. Today, this European viticultural heritage constituted by countless varieties, biotypes cultivated and wild of vines represent the biodiversity present in our territory. This biodiversity is a popular way to describe the diversity of life on our planet. The genetic diversity in agriculture can adapt to different environmental conditions speculations agricultural and socio-economic conditions (Menini, 1998).

It is very important to preserve the biodiversity for this adaptability of the vines that are not selected or those ancient vines that are no longer cultivated.

Sicily is still one of the few richest regions across Europe in viticultural biodiversity and here it's possible to find lots of vines. However, this biodiversity is increasingly under threat by development models that dominate the world scene for over a century.

The model of agricultural development established after the Second World War plays a fundamental role: the "variability productive," the promiscuous cultivation, the small plot of land, the presence of non-cultivated areas considered "unproductive", have been seen for decades as limiting factors for production and high yields, considered the main objective of farming.

Small farmers, upholder for centuries of tradition and of world's agricultural heritage, have been gradually marginalized from the process agricultural "industry". The result was the gradual selection of plants vulnerable to any environmental changes or new adversities (Menini, 1998).

The scientific community as FAO Panel of Experts on Plant Exploration and Introduction (1965), International Board for Plants Genetic Resources (1974), European Cooperative Programmers Conservation and Exchange of Crop Genetic Resources (1980) and Convention on Biological Diversity (1992) have launched several strategies to oppose this progressive erosion of the genetic heritage. The principal strategies are:

• the establishment of a worldwide network of "gene banks" and botanical gardens;

• the preservation in situ of specific heritage and varietal.

The programs for the conservation and enhancement of biodiversity at the local level can be an important response to the progressive impoverishment and degradation of the environment, contributing to the achievement of those key objectives for sustainable development to be transmitted to future generations.

At the end of 1800s, in the viticultural sector, the problem of sorting ampelography platforms was born. In the early 1700s there was a wine production of excellent quality, but since this time forward, the spread of consumption linked to the energy value of the wine was dominated by a rural viticulture, that was brought in territories not particularly suited to involve the use of poor-quality vines (Calo, 2004). In contrast to this decay an important cultural movement, supported by the Academies and a whole class of scholars (Fourth, Ridolfi, De Blasais, Oudart, Foëx, Molon, Rovasenda, Mondini, Frijo), which set out the objectives of the study and creation national ampelografic platform and of the viticultural inventory, developed. So, in 1882 the Ampelographic Central Committee and Ampelographic Provincial Commissions was founded; we can find ones in Sicily, in the provinces of Catania and Palermo (Bica, 2007). However, the commission Ampelographic clashed with the devastation caused

by the invasion of phylloxera which, together with powdery mildew and downy mildew, changed the European viticulture.

This made it necessary, in the early 1900s, a reorganization and a chart of varieties with the creation of the Register of vine varieties in the Experimental Institute for Viticulture of Conegliano, in the Veneto region.

Later, with the birth of the European Economic Community the classification of vine varieties, that can be cultivated administrative unit, was established, (EC Reg 816/70). In Italian territory the cultivation of grape vines enrolled in the National Register of variety and distinct from each province was only permitted (EC Regulation 2005/70).

In such a register 445 varieties, of which more than 300 are native, were recorded, although many of these were little cultivated. In fact, with the globalization of the market and the consequent standardization of tastes, there has been a globally decline in the number of grape varieties cultivated in favour of a few that have found wide spread because of their easy adaptability to different climatic conditions (Tosi and Mirandola, 2006).

The significant reduction of the area planted with vine varieties of minor importance has led to the disappearance of many traditional varieties and consequently the reduction of the variability within the species (Muganu *et al*., 2008).

In summary we report a summary of the different motivations of the erosion of wine heritage (Bandinelli *et al*., 2005):

- Coming of powdery mildew (1845), phylloxera (1868) and downy mildew (1878).
- Change from an economy based on self-sufficiency in exchange economy.
- Appeal to the practice of grafting on American vines;
- Viticultural reconstruction and development of mechanization in viticulture;
- Switching from mixed to specialized viticulture.
- Birth of the disciplinary production of quality wine
- Intensification of clonal selection.
- Establishment of "National Catalogue of Varieties" (DPR 24-12-1969 n.1164).
- Plans wine production (EAGGF Regulation 25/1962 and Regulation EEC 728/70).
- National Catalogue of Varieties recommended and authorized by Province

Around 1970 we understand the importance of recovery, assess and enhance the germplasm of different species. In fact projects involving the rediscovery of ancient

cultivars, varieties no longer grown, began to be created. The study of these varieties has allowed and will allow to enrich the National Register of Grapevine Varieties, as well as a source of genes for future studies of genetic improvement.

Although the majority of vines once planted was lost, you can still say that Italy preserves a great biodiversity in each region and, according to the data recorded in the National Catalogue of Varieties of Vine, has a large number of local species. The reasons that support such data are readily identifiable. Italy, in fact, has for centuries been the centre of the exchange of a wide variety of agricultural products; temperate climate is also extremely favorable to the cultivation of *Vitis vinifera* L. and the Sicilian geomorphology. It is clear, therefore, as the fundamental problem of our country is to first recognize and then preserve the magnificent and large genetic viticultural diversity.

For this purpose the European project GrapeGen06 (2006-2010) continued the process already undertaken within the program GENRES CT96 No 81 (1997-2002) with the objective of standardizing the procedures for genetic analysis leading to the creation of a European database for the vine (European Vitis Database - http://www.eu-vitis.de/index.php), which collects morphological and genetic profiles of numerous accessions preserved in Europe. At national level the AGER project (2010-2013) is contributing to the creation of an Italian wine database to be of public access for cultivars of our territory (http://www.vitisdb. en /).

At a regional level, Sicily is the largest island in the Mediterranean and presents 128,000 hectares of vineyards, more than any other Italian regions (Anderson, 2006). Paleobotanical discoveries of the genus *Vitis* reveal the cultivation of vines since the age ancient history (Collesano, 1998).

The great complexity of local cultivars in this region can be attributed to various reasons, such as: various environmental conditions within the island, cultivation of new genotypes introduced by human migration (Dangl *et al*., 2001), genetic mutations and pollination crossed with wild populations or from the domestication of wild plants (Snoussi *et al*., 2004). What is certain is that the Sicilian grapevine germplasm has been introduced at different times and from different geographical areas (Carimi *et al*., 2010).

The variability of the grapevine can be observed in terms of morphology and quality (Alleweldt and Possingham, 1988). The ampelography (from the Greek ampelos, vine and graphos, description) is useful in the identification of grape cultivars (Galet, 1991; OIV, 2009).

Unfortunately, these determinations are based on characteristics that can be affected by environmental conditions (Levadoux, 1956; Tessier *et al*., 1999).

Traditionally, morphological and agronomic characteristics were the main criteria to differentiate grape varieties, but it is known that many characters are strongly influenced by environmental conditions. However, the great variability recommends the use of more accurate methods, and a wide range of biochemical and molecular markers have been used with success to characterize and classify the germplasm collections (Calò *et al*., 1994).

Clonal selection, recovery and conservation of biodiversity, wine production quality are the key words of the project "Valorizzazione dei Vitigni Autoctoni Siciliani" (Enhancement of native grapes Sicilian), which the Department of Agriculture and Forestry of Sicily launched in 2003. The objectives of this plan are:

- Renovation and development of Sicilian grapes;
- Study and evaluation of the resource quality of the vines in relation to the "terroir";
- Obtaining clones of grape varieties approved;
- Offer grape growers to propagation material selected;
- Study of the effects on health and food safety of Sicilian wines

The objectives of the project are to promote and enrol the most significant Sicilian cultivar in the National Catalogue and the ancient vines "relic" through protocols wine can enhance the quality of the grapes.

To this end, we examined carefully the platform ampelography of Sicilian investigations involving about 500 companies located in 84 municipalities in eight provinces. This large number of companies was selected on the basis of the presence of vineyards ancient constitution where the variability is usually best preserved and where a large number of varieties in extinction was identified. The Sicilian viticultural heritage was such classified into three categories in order to activate specific initiatives for the enhancement of each grape variety:

- Grapes of regional interest;
- Grapes of local interest;
- Grapes ancient "relic".

1.2 - TERROIR

The cultivation of grapes and production of wine is of significant cultural heritage and its impact on the economies is significant.

Terroir has been acknowledged as an important factor in wine quality and style, particularly in European vineyards (Falcetti 1994). It can be defined as an interactive ecosystem, in a given place, including climate, soil, and the vine (rootstock and cultivar) (Seguin 1988). Some authors also include human factors such as viticultural and enological techniques in their definition of terroir (Seguin 1986). It is difficult to study the effect of all the parameters of terroir in a single experiment. Many authors have assessed the impact of a single parameter of terroir on grape quality: climate (Winkler et al. 1974, Huglin 1978, Gladstones 1992), soil (Seguin 1975, van Leeuwen and Seguin 1994), cultivar (Riou 1994, Huglin and Schneider 1998), or rootstock (May 1997). Wine is a complex mixture, which is the product of the interaction between the environment and several organisms' biochemical profile, including the genome, proteome, and metabolome and understanding the nature of these complex interactions has the potential to significantly contribute to improvement of viticulture and oenology.

Before introducing the results obtained in this work, some knowledge that the scientific world has provided about the flavonoids of *V. vinifera* and particularly on proanthocyanidins (tannins) will be presented.

1.3 - GRAPE PHENOLIC COMPOUNDS

Grapes polyphenols are made up of monomeric and polymeric molecules belonging classes of flavonoids and non-flavonoids. They are located in the juice (acids hydroxycinnamic related tartaric), the solid part of the pulp (proanthocyanidins, hydroxybenzoic acids), seeds (gallic acid, monomeric catechins, procyanidins oligomeric and polymeric) and skins (anthocyanins, monomeric catechins, procyanidins and prodelphinidins oligomeric and polymeric, flavonols, dihydroflavonols, hydroxycinnamic acids related tartaric, hydroxybenzoic acids, hydroxistilbenes) (Fig.1).

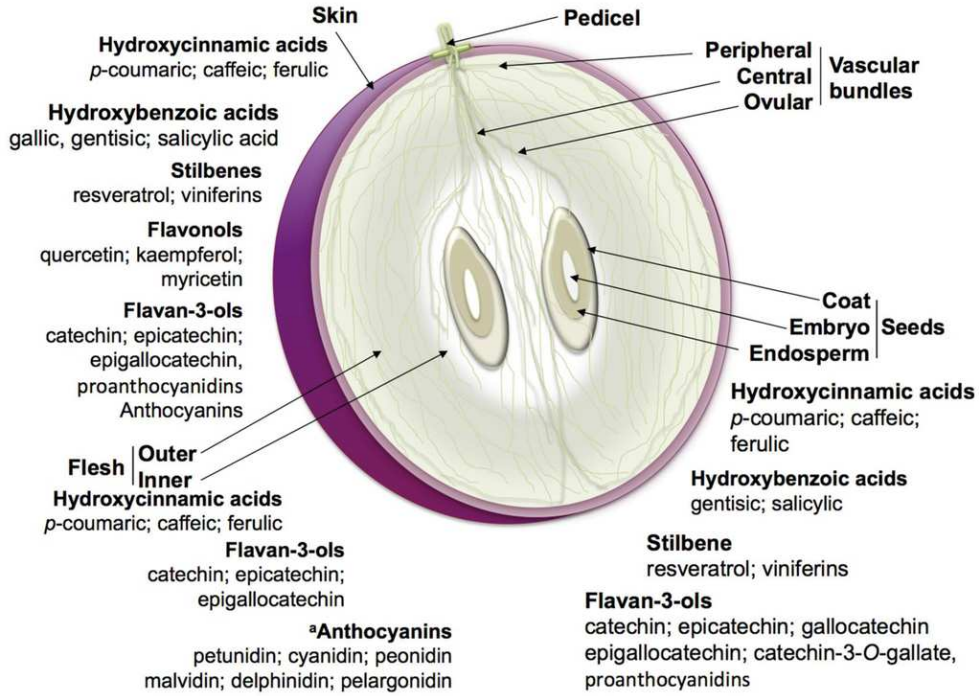


fig. 1 – schematic structure re of a ripe grape berry and pattern phenolics biosynthesis distribution between several organs and tissues (Teixeira et al. 2013)

The composition of the different classes of berries phenolic compounds is based on different factors, environmental, cultural and the level of maturity of the grapes.

In the different varieties of *Vitis vinifera*, at the level of juice, the differences are mainly quantitative. Prevails, in fact, always, caffeil tartaric acid (caftaric acid, CTA) on p-coumaroyl tartaric acids (coutaric acid, p-CUTA) and tartaric ferulil (fertaric acid, FTA). Cinnamic acid in the skins main may be the CTA or the p-CUTA, while the FTA, as in juice, is poorly represented. The solid pulp the main compounds are proanthocyanidins but their concentration for grape is always much lower than what is found in the seeds and skins.

The flavonols of the peel are glycosides of campferol (an OH in position 4' of the lateral ring), quercetin (two OH in 3' and 4 ') and myricetin (three OH in 3', 4 'and 5'), but also the laricitrin (two OH in 3' and 4' and a OCH_3 in 5'), the siringetina (a OH in 4' and two OCH_3 in 3' and 5'), the isorhamnetin (a OH in 4 'and a OCH3 in 3 ') have been reported (Cheynier and Rigaud, 1986; Mattivi et al., 2006; Castillo-Munoz et al., 2007).

Myricetin is basically absent in the skins of white grapes (Di Stefano et al., 2002). Forms glucoside and glucuronide flavonols generally prevail over forms galactoside, ramnoside and disaccharide (Cheynier and Rigaud, 1986). Colored grapes in the synthesis of quercetin and myricetin and probably other flavonols, are under the control variety (Squadrito

et al., 2007); the campferol is always underrepresented (Di Stefano et al., 2002; Mattivi et al., 2006).

Anthocyanins in the grapes skin are 3-glycosides of cyanidin, the peonidin, the delphinidin, the petunidin and malvidin. The position 6 of glucose can be esterified by acetic acid, p-coumaric and and from caffeic acid (Wulf and Nagel, 1978). The synthesis of the individual molecules of anthocyanins and the degree and type of acylation are under the control varietal (Castia et al., 1992).

The flavanol monomers ((+)-catechin, (-)-epicatechin, (-)-epicatechin gallate) and oligomers (procyanidins especially dimers and trimers) are in the seeds so significantly greater than the peel. The majority of the molecules of this class is found in grapes as the polymer. In the skins of their synthesis (report prodelphinidines/procyanidins) it is under the control varieties. Interesting is, finally, the presence of hydroxistilbene (resveratrol, iceatannolo, pterostilbene, viniferins) contained in grapes prevalently in the form glucosylated. Their synthesis, as well as be induced by elicitors biological and chemical is up to control varietal (Bavaresco and Fregoni, 2001).

1.4 - BIOSYNTHESIS OF POLYPHENOLS

Polyphenols are made of two amino acids aromatic, phenylalanine and tyrosine. They have generated since the simple sugars of primary metabolism across the schikimic acid pathway (fig. 2)

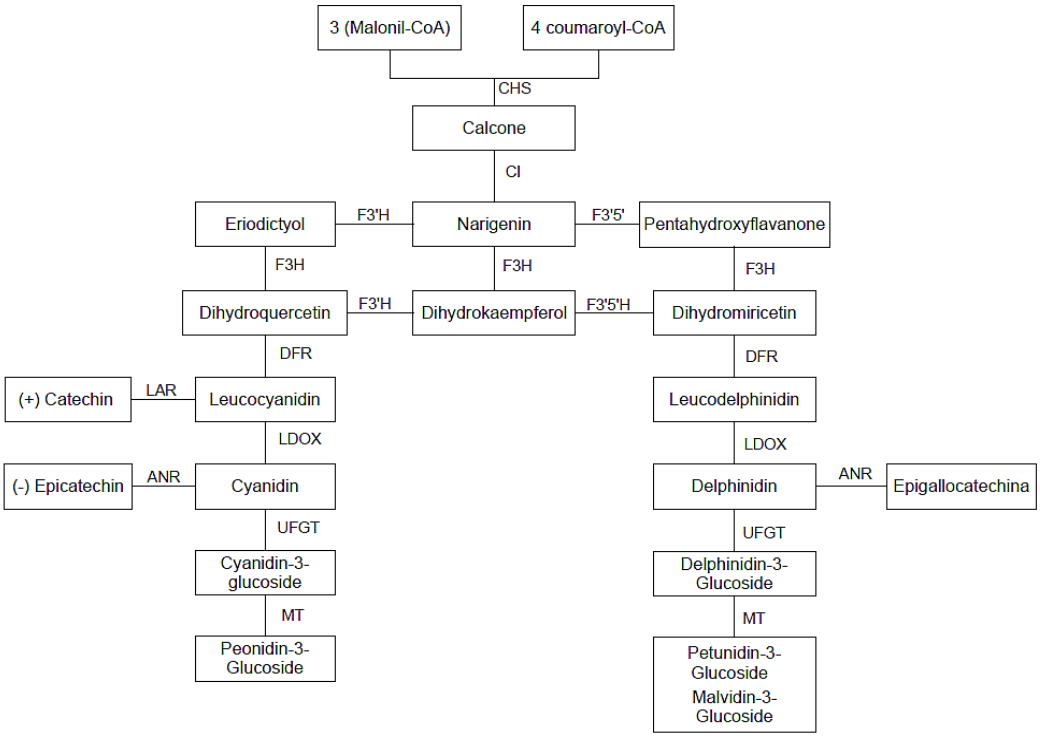


Fig. 2 – biosynthetic pathways of berry polyphenols

By phenylalanine (giving rise to the biosynthetic pathway of phenylpropanoids), the phenylalanine ammonialyasi (PAL) produces cinnamate molecule not yet enol, which allows the formation of hydroxycinnamic acids: p-coumaric acid (thanks to the cinnamate-4-hydroxylase or C4H), caffeic, ferulic and sinapic. Forms then cumaroil-CoA by the action of coenzyme A ligase (4CL). From this intermediate the biosynthetic pathways of the major classes of polyphenolic compounds are born. They therefore make:

- The benzoic acids of the series for β-oxidation. The hydrolysable tannins (gallic and ellagic); are coming from the combination of simple sugars with gallic acid (Lee and Raskin, 1995 Macheix et al., 2005);
- The esters of hydroxycinnamic by hydroxylation of p-coumaric acid and subsequent esterification with an alcohol acid. Examples: hydroxycinnamic acid, chlorogenic acid, tartaric esters;
- Coumarins for internal cyclization of the molecules;
- Stilbenes (in particular resveratrol): they are produced from the stilbene synthase (StSy) that competes for the same substrates with chalcone synthase;

- The flavonoids, in the form of chalcone biosynthetic pathway that has an origin, mixed: from one side 3 molecules of acetyl-CoA for the cycle A, the other one molecule of p-coumaroyl-CoA to the cycle B and the heterocycle C (Macheix et al., 2005). Starting from chalcone, the biosynthesis of flavonoids requires the sequential action of the enzymes chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), dihydroflavonol reductase (DFR), and leads to the formation of leucoantocianidine.

These substances are the precursors of anthocyanins, through the sequential action of leucoantocyanidin dioxygenase (LDOX) and UDP-glucose: flavonoid 3-O-glucosiltransferasi (UFGT). The flavonoid synthesis is further supplemented by enzymes that modify the phenolic B ring, inserting hydroxyl groups and methyl. In particular, inside the berry, anthocyanins are commonly methylated: in positions 3' (peonidin and petunidin) thanks to the 3'-O-methyltransferase (3'OMT); in positions 3', 5' (malvidin) thanks to the 3', 5'-O-methyltransferase (3', 5'OMT).

From the dihydroflavonoli, flavonols are produced as per flavonol synthases action (FLS), 2,3-trans-leucoanthocianidins per share of dihydroflavonol reductase (DFR). From the 2,3-trans-leucocianidina originates (+)-catechin and the 2,3-trans leucodelfinidin (+)-gallocatechin thanks to leucoanthocyanidin-reductase (LAR) or anthocyanidins (cyanidin and delphinidin). A anthocyanidin reductase produces 2,3-cis flavanol, (-)-epicatechin and (-)-epigallocatechin. Few authors were thinking that the condensed tannins (proanthocyanidins), are coming from the condensation of leucoanthocianidins or catechins mixt by an enzyme condensing (EC) (Schubert et al., 2003) have not yet been identified; in fact, the synthesis of these molecules has not yet been rationalized.

Metabolism phenolic enzymes. The adjustment biosynthetic enzymes follows a well-defined model linked to the development. Most of them (CHS, CHI, F3H, DFR and LDOX) are already activated by bloom and show a peak in the next few weeks and then later after veraison, while the enzyme UFGT has only the second peak activation in correspondence of the latter stage where the anthocyanins are accumulated in the grape skin (Schubert et al., 2003).

• PAL: phenylalanine ammonialyase allows the formation of cinnamic starting from phenylalanine and regulates quantitatively the accumulation of phenolic compounds. It has two activity peaks: the first during the growth of berry (perhaps

linked to the synthesis of hydroxycinnamic acids, flavonols and flavans), then decreases; another increment veraison to maturity, then a decrease in the final. The activity is inhibited by high temperatures and stimulated by light.

• CoA ligase: allow unions hydroxycinnamic acids with CoA. The different enzymatic shape are specific to the various phenolic compounds and direct-CoA esters to the various ports.

• CH4: cinnamate 4-hydroxylase activity presents the maximum maturity and then a decrease.

• CHS: chalcone synthase allows you to start off the flavonoids, by condensation of phenylpropanoids. The activity is not detectable prior to coloring (perhaps due to poor flavonoid synthesis during growth the berry), exhibits a maximum at maturity and then rapidly decreasing.

• WHO: chalcone isomerase follows a trend similar to PAL.

• UFGT: anthocyanin glucose transferase reaches maximum activity at maturation (Schubert et al., 2003).

1.5 - FACTORS THAT REGULATE THE BIOSYNTHESIS

The **genotype** is crucial for the levels of phenolic compounds. The difference of concentration is due to climatic factors and are far lower than those due to genotype. Then prevail variations due to cultivar than due to the vintage, within the same variety.

The **light**: is among the most important factors for the metabolism phenolic, especially for the accumulation of anthocyanins and flavonols. The light provides the energy for photosynthesis and stimulates the metabolic processes; its saturation point is about 700 μE/m/s. The light leads to the induction of the synthesis of various enzymes, but especially the PAL (photo inducible enzyme system), which leads to the transcription of the m-RNA, and the formation of enzymatic proteins. A similar effect is obtained with the CHS and the other enzymes of the metabolism of flavonoids, which lead to the accumulation of these compounds. The redox state of the cell and, in particular, its content in glutathione, plays an important role in activation of the intracellular transduction via, starting from perception of the light signal to the expression of the gene of the CHS.

The **temperature** is important because it directly affects the development of the berry. Temperatures of 20-25 °C are optimal for herbaceous growth, higher temperatures prevent the cells multiplication. During the maturation, when the thermal requirements are around 20 °C, the temperature has a larger impact on the translocation of sugars. Generally extreme conditions have negative effects on the intensity coloring: temperatures higher than 37°C inhibit the formation of anthocyanins, while the lower ones are slowing down the synthesis. The interaction between temperature and light are interesting in that it is believed that a drop in temperature associated with a bright adequate treatment induces the accumulation of anthocyanins.

While the first exposure experiments were primarily concerned with the impact of light on anthocyanin accumulation in the grape, other flavonoid components such as flavonols and tannins have also attracted research interest. Flavonol accumulation in plant tissues has previously been studied in a number of species, with exposure to UV shown to increase flavonol glucosides in vegetative and reproductive tissues (Hrazdina and Parsons 1982, Ryan et al. 1998, Vogt et al. 1999, Reay and Lancaster 2001). This effect has also been reported in winegrapes, with exposed fruit higher in flavonol glucosides, while shaded fruit had lower flavonol content (Price et al. 1995, Haselgrove et al. 2000, Spayd et al. 2002). More recently, Downey et al. (2004a) reported that the level of flavonols in both leaves and fruit of the grapevine were almost negligible when those tissues had not been exposed to light. Subsequent exposure of those tissues to sunlight resulted in a rapid increase in flavonols accumulation and in expression of the gene encoding flavonol synthase (Downey et al. 2004b). In contrast, tannin accumulation in Shiraz grape berries appears to be largely unaffected by bunch exposure (Downey et al. 2004a). In the seed there was no observable effect of bunch exposure on either the proanthocyanidin content or composition. In the skin of the grape berry at harvest, there was also no appreciable difference in tannin content. However, the study examined cyanidin content and composition throughout berry development in both shaded and exposed fruit, revealing significant differences in both content and composition throughout the intermediate stages of berry development, with shaded fruit reaching a much lower maximum in proanthocyanidin content than exposed fruit. The peak in proanthocyanidin accumulation in winegrapes occurred around time of veraison and then decreased toward harvest in what is generally considered to be a decrease in tannin extractability rather than degradation or turnover (Czochanska et al 1979,

Amrani-Joutei et al. 1994, Escribano-Bailon et al. 1995, Cheynier et al. 1997b, Saint-Cricq de Gaulejac et al. 1997, de Freitas and Glories 1999, Kennedy et al. 2001, Downey et al 2003a). This decrease in tannin extractability was observed in both shaded and exposed fruit; however, the decrease was greater in exposed fruit such that the levels were virtually the same in shaded and exposed fruit at harvest. The effects of shading on tannin accumulation in grape berries have only been examined in Shiraz, although this is an active area of research in the Australian wine industry.

A moderate **water stress** stimulates the biosynthesis of anthocyanins (which adds to the concentration of the solutes and the increase of the ratio peel / pulp) (Ojeda et al., 2002), while does not influence the content of flavanols. Stressed plants accumulate more anthocyanins trisubstituted and methylated; in particular is stimulated gene UFGT, head of the accumulation of anthocyanins in berries (Castellarin and Peterlunger, 2007). Instead, an excess water leads to an increase of the volume of the berries and this reduces the concentration of the phenols (Matthews and Anderson, 1989). However, the authors considered that changes in the structure and development of the skin were responsible rather than any direct effect on flavonoid biosynthesis (Roby and Matthews 2004, Roby et al. 2004). The difficulty with interpreting deficit irrigation treatments is that water availability impacts on a wide range of plant processes apart from flavonoid biosynthesis. For example, stomatal closure in response to water deficit reduces photosynthesis, thereby reducing all metabolite accumulation and resulting in decreased root and shoot growth (Jones 1992).

Nutrient availability has a relevant influence on plant growth (Russell 1961, Marschner 1995, Keller et al. 1998) and has been shown to effect the flavonoid composition of plant tissues. Whitin the three nutrients commonly applied as fertilizer-nitrogen, potassium, and phosphate only nitrogen and potassium have thus far attracted viticultural research. Both low and excessively high levels of nitrogen fertilizer have been shown to decrease color in grape berries (Kliewer 1977, Keller and Hrazdina 1998, Delgado 2004), while high potassium has been reported to decrease color in grapes (Morris et al. 1983, Jackson and Lombard 1993). The most likely mechanism for decreasing phenolic content at high nutrient levels is excessive vigor.

Vine vigor has also been reported to impact upon the tannin content and composition of grape skins in Pinot noir. In the berry skin, proanthocyanidins were higher in low-vigor vines, with an increase in the proportion of epigallocatechin

subunits in proanthocyanidin polymers and an increase in the average size of polymers observed with decreasing vine vigor (Cortell et al. 2005). It is uncertain whether this change is due to the difference in vine vigor or is an indirect effect of changes in canopy architecture resulting in differential bunch exposure effects. Physical characteristics can also affect flavonoid accumulation (Jackson and Lombard 1993, McDonald et al. 1998). Such characters as the parent material and the age of the soil that largely determine the micronutrient pool, structure, and texture of soil have a significant effect on plant growth (Russell 1961, Northcote 1992, Marshner 1995).

However, the major consequence of soil type is the capacity of the soil to hold **water** while remaining sufficiently well-drained to avoid water logging (Russell 1961, Northcote 1992). Irrigation can alleviate water-stress-related reductions in plant growth and development, although some reports suggest water deficit increases tannin and anthocyanin content in grapes (Nadal and Arola 1995, Dry et al. 1998). In grape cell cultures, anthocyanin biosynthesis is extremely sensitive to osmotic stress (Do and Cormier 1991, Suzuki 1995). Osmotic stress results in increased anthocyanin accumulation, which suggests that deficit irrigation could be a powerful tool for managing anthocyanins in the vineyard. However, some research suggests that while excessive water application decreased tannin content (Kennedy et al. 2000), water deficit had little or no effect on tannin or anthocyanin accumulation in the grape berry (Kennedy et al. 2000, 2002, Stoll 2000). Rather, the primary effect of water deficit was to decrease berry size and thus change the ratio of skin weight to total berry weight and therefore anthocyanin and tannin concentration in the berry. Closer investigation of this phenomenon suggested that changes in anthocyanin and tannin concentration did in fact occur with deficit irrigation aside from any effect related to berry size (Roby et al. 2004). However, the authors considered that changes in the structure and development of the skin were responsible rather than any direct effect on flavonoid biosynthesis (Roby and Matthews 2004, Roby et al. 2004). The difficulty with interpreting deficit irrigation treatments is that water availability impacts on a wide range of plant processes apart from flavonoid biosynthesis. For example, stomatal closure in response to water deficit reduces photosynthesis, thereby reducing all metabolite accumulation and resulting in decreased root and shoot growth (Jones 1992). In extreme cases this may lead to senescence of some tissues and alter source-sink relationships within the plant (Coombe 1989). Many such responses are regulated by plant growth regulators such as

abscisic acid, ethylene, cytokinins, gibberellins, and auxins, and the influence of these compounds has been specifically examined with respect to their influence on flavonoid biosynthesis.

Hormones: actively intervene in the regulation of gene expression of the biosynthesis of phenolic compounds (gibberellins/cytokinins). These act on the MYB transcription factors, which are the regulators of the metabolism phenolic. Cytokinins strongly stimulated the accumulation of anthocyanins. Gibberellins, a more stimulating the accumulation of anthocyanin in the presence of sucrose, essentially used as a carbon source. Also ethylene stimulates metabolism phenolic, with an increase in the PAL (Macheix et al., 2005). Abscisic acid induces accumulation of anthocyanins in the berries, after veraison only, that is when is active most of the genes (Schubert et al., 2003). In grapevines, abscisic acid has been shown to increase anthocyanin accumulation in grape berries of the cultivars Olympia, Kyoho, and Cabernet Sauvignon (Matsushima et al. 1989, Hiratsuka et al. 2001, Ban et al. 2003, Dan and Lee 2004, Jeong et al. 2004). The application of gibberellins (GA3) to grapes has generally been reported to decrease anthocyanin levels in the fruit (Dan and Lee 2004). Gibberellic acid is usually applied to table grapes to increase berry size and the decrease in anthocyanin levels under these circumstances is likely to occur through an effective dilution of anthocyanin concentration in individual berries. A similar effect has been reported with the growth regulator forchlorfenuron (N-(2-chloro-4-pyridyl)-N'-phenylurea; CPPU) (Dan and Lee 2004). Auxins and cytokinins are also plant growth regulators that were extensively used to manage plant production. While these were shown to increase anthocyanin biosynthesis in plants generally (Deikman and Hammer 1995, Nakamura et al. 1980, Ozeki and Komamine 1981), the application of the auxin napthaleneacetic acid was shown to inhibit the anthocyanin accumulation in Cabernet Sauvignon grape berries (Jeong et al. 2004). Application of the synthetic auxin benzothiazole-2-oxyacetic acid to Shiraz vines delayed the onset of ripening, including anthocyanin accumulation in the grape skin (Davies et al. 260 – Downey et al. 1997), a result of a delay with regards to of genes encoding critical steps in anthocyanin biosynthesis such as chalcone synthase and UFGT. A similar result was reported in the Kyoho cultivar with auxin 2,4-dichlorophenoxyacetic acid inhibiting anthocyanin accumulation and expression of the flavonoid pathway genes phenylalanine ammonialyase, chalcone synthase, chalcome isomerase, dihydroflavonol reductase, and UFGT, while the same genes were

upregulated and anthocyanin content increased with the application of abscisic acid (Ban et al. 2003). Ethylene has also been identified as a plant growth regulator that has a particular effect on fruit ripening (Burg and Burg 1965). Application of ethylene had no effect on anthocyanin accumulation in Arabidopsis (Deikman and Hammer 1995); however, in grape berries there seems to be a requirement for low levels of endogenous ethylene for anthocyanin biosynthesis (Chervin et al. 2004). Exogenous application of ethylene or ethylene-releasing compounds has also been shown to enhance grape skin color (Roubelakis-Angelakis and Kliewer 1986, El-Kereamy et al. 2003).

The elicitors: are molecules produced generally by plant pathogens that activate the genes responsible for plant defense, inducing the production of molecules with antifungal activity, also known as phytoalexins.

Among the polyphenols, the coumarins, stilbenes, the isoflavones, etc can be identified. So a pathogenic microorganism, or an elicitor, can stimulate the production of phenolic compounds (and the enzyme PAL) by the plant. The PAL plays an important role in signal transduction within the cell.

1.6 - POLYPHENOLIC ACCUMULATION

The berry development of Vitis vinifera L. follows a trend of growth represented by a curve in the double sigmoid (Fig. 3). The cell multiplication takes place in the first phase, then stops during the veraison, and finally resumes during ripening following a cell expansion. Veraison is the most important time: the pulp becomes softer, accumulate hexose sugars (glucose and fructose), decreases the content of the acids (especially the malic acid), aromatic compounds are produced, the epicarp is colored and will have different modifications concerning the concentration of the phenolic compounds contained in the different parts of the berry. In addition, the grape, not climacteric fruit, at this time accumulates high amounts of abscisic acid while decreasing the auxin (Schubert et al., 2003).

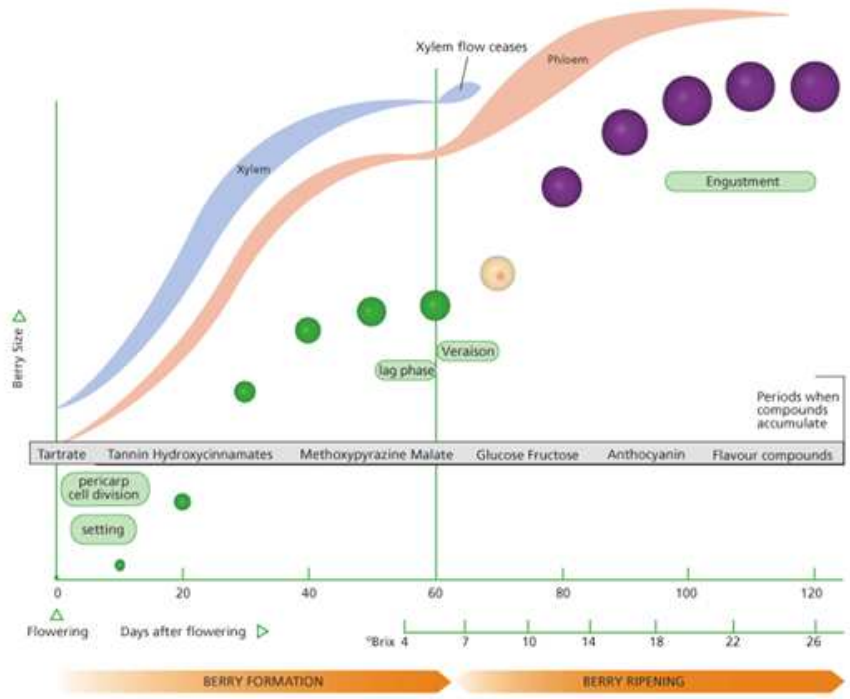


Fig. 3 - berry growth cycle

The examination of the accumulation of polyphenols, realizes the analytical results of the checks made during the ripening of the grapes. Since the establishment the berry are synthesized proanthocyanidins, the HCTA and flavonols. There no enough information about the evolution of the constituent monomers of proanthocyanidins peel. It appears that the synthesis of (+)-catechin, the (-)-epicatechin, the (-)-epicatechin gallate and (-)-epigallocatechin start in parallel. It seems clear, however, that at the veraison the synthesis of these compounds or proceeds slowly or undergoes a crash. In any case it is not very smooth, probably due to the great variability that is found at the level of the berries.

The tannins of the skins are summarized in the green phase and their concentration is highest at veraison. They have complex structures, but their degree of polymerization varies little. The concentration of dimers and trimers present veraison decreases slightly during ripening. Moreover, as you get closer to maturity, the skin tannins are always less reactive towards proteins and, consequently, reduces their aggressiveness and astringency. This phenomenon is explained by the progressive increase in the degree of polymerization, which in maturity can reach from 15 to 40 mDP, but the absolute content remain constant or decrease (Kennedy et al., 2001).

The tannins of the seeds, which are present in high numbers at veraison, decreased from this stage the berry growth to maturity. The decrease is more or less relevant

according to the maturity and in connection with the accumulation of the anthocyanins in the skins. The tannins of seeds are of procyanidins whose average degree of polymerization (mDP) is low at veraison and remains always lower than 10 during maturation (Kennedy et al., 2000a). Proanthocyanidins of grape seeds are molecules with different properties.

Proanthocyanidins of grape seeds are molecules with distinct tannins. The concentration of tannins extracted from grape seeds generally tends to decrease during the ripening of the grapes, as the outer skins of the seed undergoes oxidative browning, which causes a major fixation of tannins to the epithelial cells. This decrease is more or less important depending on the conditions of ripening, so vintage. The reduction also varies as a function of the grape (Kennedy et al., 2000a). These are molecules in the free state, not colloidal and highly reactive in respect of proteins.

The tannins of the stalks are significantly represented already veraison and vary little during ripening. Are polymerized procyanidins, non-colloidal, with a reactivity similar to that of the tannins of the seeds. Overall it can be seen that the skins are particularly rich in complex tannins-polysaccharide-protein and tannins that give a delicate flavor. On the contrary, the stems and the seeds are characterized by the abundance of polymeric procyanidins conferring astringency more aggressive.

Close to veraison is also observed a decrease of the content of HCTA of the flesh and skin which could indicate a shutdown of their synthesis, their degradation or their use in the synthesis of other compounds, eg. anthocyanins p-cumarati, in the skin. This decrease can be affected by the increase in volume of the berry.

From veraison, in fact even a few days before, begins the synthesis of anthocyanins, is diverted towards which the majority of the metabolites of flavonoid synthesis. The synthesis of anthocyanins continues actively immediately after veraison and decreases in intensity near the ripening of the fruit; ripened it is observed a slight decline. The evolution of the concentration in anthocyanins and tannins in the skins, is valid for all varieties and for most wine regions, but the level and trend of accumulation can vary greatly depending on the genetic basis, the climatic and vintage.

The synthesis of flavonols begins long before veraison, already in flower. In the early stages clearly prevails synthesis of quercetin; that of myricetin starts very slowly, then close veraison can prevail in intensity on that quercetin in varieties in which, at the level of anthocyanins, the prevailing pathway of ampelopsin (Squadrito et al., 2007).

Varieties where the prevailing way of dihydroquercetin, the content of quercetin always exceeds the myricetin.

On the progress of individual anthocyanins, near veraison, in compare to later periods, there was an increase faster molecules disubstituted. The anthocyanin profile varietal, however settles immediately after veraison and undergoes little change during ripening varieties to prevalence of anthocyanins trisubstituted ring side; some variation, was observed in those with a prevalence of anthocyanins disubstituted. The anthocyanin profile of the latter is affected much more than those with a prevalence of molecules trisubstituted, by environmental variables (Di Stefano et al., 1994). The presence of anthocyanins in the grape skin polymer has been reported by Kennedy et al. (2001), Kennedy et al. (2002) and Canals et al. (2005). The structure of these pigments is still hypothetical, even if it were acceptable, in the process just mentioned that it is of molecules in which one part is constituted by a part flavanols and polysaccharides.

Vidal et al. (2004a) established the structure of anthocyanins trimers present in grape extracts. It is trimers with C-C bonds 4-8, type procyanidins B type, and with C-C bond C-O-C 2-7 4-8 and the type of proanthocyanidins of type A. The shape flavilio anthocyanin is in the drive terminal. These molecules have a certain resistance to discoloration with SO_2 and resistant to hydrolysis by acid catalyzed by heating in an acid solution for strong acid. The formation of polymeric pigments would be the degree of ripening (Fournand et al., 2006). According to data reported by these authors, the curves of evolution of the anthocyanin pigments total monomers and during the ripening of the grapes diverge especially from about 200 g/L of sugar when the content of anthocyanins monomers begins to decrease, while that of total anthocyanins increases slightly and then decreased from about 240 g/L of sugar.

The determination of the polyphenol monomers and polymers in grapes is of particular importance in making (choosing the right time for the harvest, phenolic maturity) and in the study of the synthesis of the different classes of polyphenols in individual parts of the berry (pulp, seeds and peel).

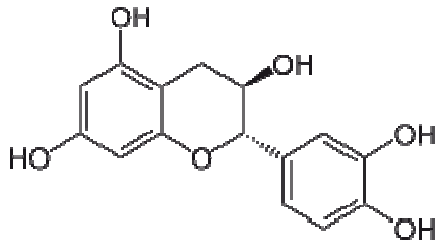
They are defined tannins those substances able to give rise to stable combinations with proteins and other vegetable polymers (Ribereau-Gayon et al., 2003).

One of their characteristics is their certain properties of precipitate proteins (Haslam, 1998). The tannins also interact with a wide range of other biological molecules including polysaccharides and alkaloids (Haslam, 1998). This property has

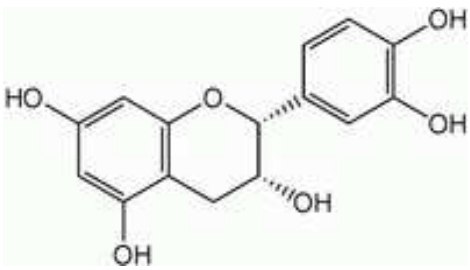
been used with success in the excess removal of tannins in the wine and also has been adapted for the measurement of the tannins in the wine. (Montedoro and Fantozzi, 1974 Hagerman and Butler, 1978; Harbertson et al., 2003; Sarneckis et al., 2006).

The flavan-3 oils, based on their chemical structure, are considered as precursors of tannins, but the way that allows the interflavanic bonds remains unknown (Adams, 2006). The compounds overrepresented among flavan-3-ols or flavanols are the (+)-catechin and (-)-epicatechin (constitutional isomers); in grapes you are also found the (+)-gallocatechin, the (-)-epigallocatechin and (-)-epicatechin gallate (fig.4). The flavan-3-ols monomers are produced prior to coloring and increase during maturation. In this class of polyphenols are present very different biomolecules, such variability is linked to the size of the compounds. The molecular masses of the active tannins are generally comprised between 600 and 3500.

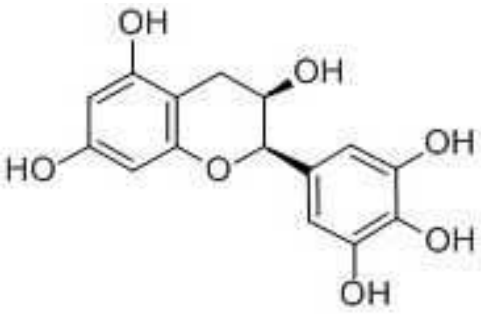
Based on the nature of the elementary molecules are distinguished tannins gallic hydrolysable or condensed tannins or catechins. The hydrolysable tannins include gallotannins and ellagitannins, substances that are not in the grapes. The condensed tannins are composed of polymers formed from sub-units with extensions and multiple terminals that are structurally similar to the flavan-3-ols (Haslam, 1998). In the seeds the (+)-catechin, the (-)-epicatechin and (-)-epicatechin gallate, in the skins to the (-)-epicatechin and (-)-epigallocatechin represent the basic monomeric units.



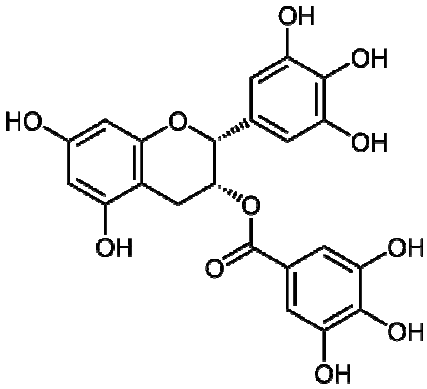
(+)-Catechin (C)



(-)-Epicatechin (EC)



(-)-Epigallocatechin (EGC)



Epicatechin-3O-galate (ECG)

Fig. 4 - Structures of flavan-3-ols

The simpler compounds belonging to the class of tannins are the dimer procyanidins (Fig 5), which can be divided into two groups: B-type dimers procyanidins resulting from the condensation of two units of flavan-3-ols linked together by means a bond C4-C8 (B1 to B4) or C4-C6 (B5 to B8), and those of A type, which in addition to possessing a interflavanic bond C4-C8 or C4-C6 possess an ether bond-type between carbons C5 or C7 of the terminal unit and the carbon C2 of the upper unit.

Trimers procyanidins can be divided into two categories: the procyanidin C-type trimers in which two interflavinic bonds may be of C4-C8 and/or C4-C6, and D type trimers in which a link can be interflavanic type C4-C8 or C4-C6 while the second is of the A type.

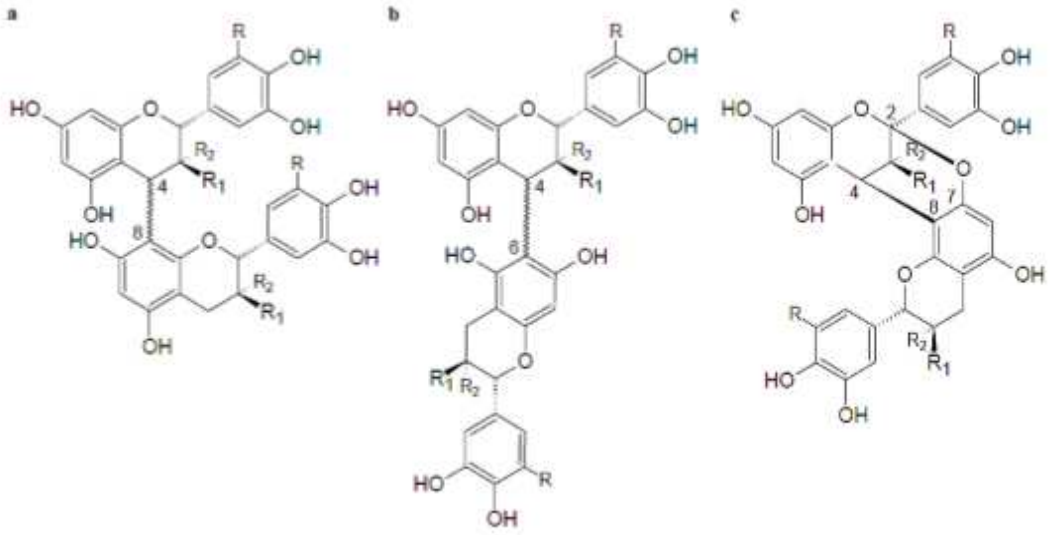


Figure 5 - Structure of dimers procyanidins B type and A type. A: procyanidins B1-B4; b. procyanidin B5-B8; c. A procyanidin.

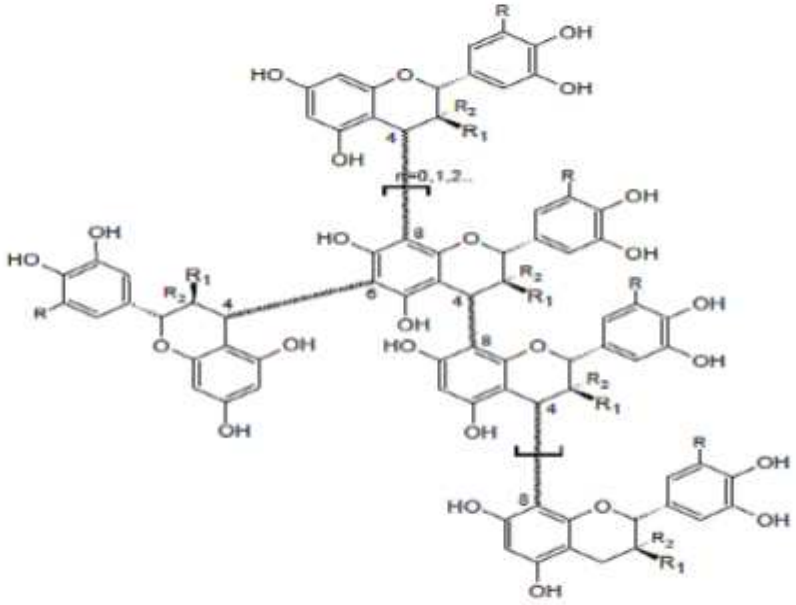


Figure 6 - Structure of condensed proanthocyanidins

Based on the number of units flavaniche finally distinguish the oligomeric procyanidins (from 3 to 10 units) from the condensed procyanidins (more than ten units) (fig. 6), that have a molecular mass of more than 3000 (Rib'ereau-Gayon et al., 2003).

The tannins of the skins are very different from those of the seeds for both the composition of the monomers in the monomers and extension terminals, both for the average degree of polymerization (~ 30 for the first, for ~ 10 seconds).

Seeds between monomeric catechins, can prevail (+)-catechin or (-)-epicatechin and dimers procyanidins between the B1 or B2. The monomers terminals are represented by (+)-catechin, less from (-)-epicatechin and (-)-epicatechin gallate (Downey et al., 2003).

Among the flavanols skins prevail polymers on oligomers, while the monomeric catechins are underrepresented (Mateus et al., 2001). The proanthocyanidins of the skins are characterized by the prevalence of (-)-epicatechin and (-)-epigallocatechin (absent in the seeds) on the (-)-epicatechin gallate and (+)-catechin between the extension unit (Souquet et al ., 1996; Pastor of Rio and Kennedy, 2006). The latter, however, may represent the majority of terminal units (Souquet et al., 1996; Downey et al., 2003). Have been reported between the extension unit small amounts of (+) - gallocatechin and (-)-epigallocatechin gallate (Souquet et al., 1996; Downey et al., 2003). The relationship between the levels of (-)-epigallocatechin and (-)-epicatechin of the extension units of proanthocyanidins of the skins appears to be under the control varieties.

1.7 - TANNINS AND THE SENSATION OF ASTRINGENCY

The tannins, including condensed tannins derived from grapes, produce kind of astringency in foods and beverages and contribute to the "structure" or "body" of red wine.

The term astringency refers to dryness and to a feeling of wrinkling in the mouth (Lee and Lawless, 1991) and is a characteristic of red wine (Kennedy et al. 2006; Gawel et al. 2001). The quality of the tannins of the wine depends on the maximum intensity of the sensation in the mouth, the total duration and the time taken to reach the maximum intensity (Kallithraka et al. 2001), the roughness and the degree of dryness of the mouth (Lee and Lawless , 1991; Kallithraka et al. 2001; Demiglio and Pickering, 2008).

The sensations of astringency of the wine are considered pleasant when balanced with other factors, including the content of alcohol and sugars. High concentrations of tannins and acids rather than sugars produce a wine very astringent that is considered

"hard", "immature" or "green", instead, high concentrations of sugars can lead to a wine which can be described as "dense" or "flaccid" (Kennedy, 2008). The astringency influence the quality of the red wine (Boselli et al. 2004; Landon et al. 2008) and therefore knowledge of the structures of the compounds in the matrix astringent wine and the impact of these structures on sensory properties can be an important consideration on the of vinification technique.

The tannins typically tend to bind to proteins and therefore can potentially cause gastrointestinal denaturing digestive enzymes in the gut. It is believed that the salivary proteins bind efficiently to the tannins to compensate for this effect and also to act as a detection mechanism (Charlton et al., 2002; Haslam, 1996). It is believed that the aggregation of the resulting complex protein-tannin and the resulting increase in friction give rise to the sensation of astringency (Dinnella et al., 2009; Baxter et al., 1997), but these interactions are only one part of the complex feeling that may cause a range of perceptions: from a velvety texture homogeneous to a feeling harsh or crimping (Gawel et al., 2000; Hufnagel and Hofmann, 2008; Gawel, 1998).

The spectrum of subtle differences in the sensations of astringency was described in the "panel of the sensations of red wine in the mouth" from Gawel et al. 2000 includes descriptors such as "dusty" up to "glue" and "aggressive".

The astringency of wine is influenced by many factors, including the structure and content of tannins (Kennedy et al., 2006), the presence of macromolecules such as polysaccharides (Vidal et al., 2004b; Carvalho et al., 2006), residual sugars (Gawel et al., 2007), the concentration of smaller molecules, such as anthocyanins and flavanols monomers (eg., (+) - catechin) (Kallithraka et al., 1997a; Vidal et al., 2004c), acidity (Fontoin et al., 2008; Kallithraka et al., 1997b) and the ethanol content (Demiglio and Pickering, 2008; Fontoin et al., 2008).

1.8 - THE TANNINS OF THE RED WINE

The tannins in red wine are made from condensed tannins derived from grapes and structurally modified during winemaking. A small percentage of hydrolysable tannins are extracted from the oak barrels or wood fragments (chips) during ripening and storing wine (Sarneckis et al., 2006). This compounds it self however, hardly

contribute astringency (Pocock et al., 1994). The condensed tannins of the skins are in part already extracted in the aqueous phase before the fermentation process. As the fermentation continues, the tannins begin to be extracted from the seeds also (Peyrot and Kennedy, 2003; Herderich and Smith, 2005). It was also shown that the match between the cold solid parts of the grapes and the must contributes to increase of tannins even in the absence of ethanol, which can be connected to hydration of the seeds before fermentation (Busse-Valverde et al., 2010). During fermentation, the structure of tannins extracted from grapes is altered by chemical oxidation processes and enzymatic reactions as well as from indirect condensation (Monagas et al., 2005; Cheynier, 2006; Cala et al., 2010), which are facilitated by products of fermentation and oxidation as acetaldehyde, pyruvic acid and glicossilico (Fulcrand et al., 2006; Es-Safi et al., 1999). For example, condensation reactions mediated dall'acetaldehyde may initially lead to the formation of oligomers of procyanidins with ties ethyl or pigmented polymers (Dallas et al., 1996). These may further polymerize to a colored shape tannins that are potentially more prone to bending and intramolecular bond than the linear structures of the grape tannins (Poncet-Legrand et al., 2010).

During maturation and aging, the wine constituents continue to suffer chemical changes that affect the structure of tannins. The acidic conditions and slow oxidation in wine lead to the severing of ties and rearrangement reactions (Zanchi et al., 2008), which are thought to cause the polymerization of tannins, as well as the creation of different pigments and pigmented polymers (Mateus et al., 2006; Salas et al., 2005). The tannins of old wines have a greater amount of colored anthocyanins incorporated in structure than tannins isolated from young wines (McRae et al., 2010) and this, at least to some extent, explains the decrease of concentration of anthocyanins in the wine with aging (Fulcrand et al., 2004; Mateus and Freitas 2001). It has been shown that the tannins are oxidized more intramolecular interactions, changing the conformation in solution of tannins to structures more condensed or more bent than the extended forms of tannins of the grapes (Poncet-Legrand et al., 2010). The changes in the tannic structure with the fermentation of the grape and wine aging can affect the binding of tannins with salivary proteins and therefore the astringency of the wine.

With aging the color of wine changes gradually from purple to red brick and, generally, the tannins become less astringent. The shade change is linked to the creation of more stable as the pigments visit with A and B and their derivatives, anthocyanins

from grape (Vivar-Quintana et al., 2002; Morata et al., 2006), as well as to browning oxidative (Li et al., 2008). Still it remains uncertain due to the decrease of astringency of red wine over time. They can contribute to a reduction of astringency the decrease in the concentration of tannins due to the reactions with residual proteins or polysaccharides during ripening and aging (Waters et al., 1994), the polymerization and subsequent precipitation of tannins, instead, their depolymerization. However, it is reported that some older wines have tannins in a similar concentration to the young wines (Mercury and Smith, 2008) and even the older wines are generally considered less astringent, which suggests that the structural changes of tannins may also have an impact on perception of astringency (Herderich et al., 2006). It has been shown that the tannins of the wine aged have molecular sizes greater than those of the young wine (McRae et al., 2010), a characteristic that is generally correlated with greater astringency (Vidal et al., 2003). The decrease of astringency due to aging could therefore be explained by an increase in intramolecular bonds by oxidation which would result in a reduced structural flexibility and therefore less interaction with proteins.

It has also been shown that artificially oxidized tannins have a greater hydrophobicity of natural tannins (Zanchi et al., 2008), which can also influence the effectiveness of the bonds. The micro-controlled involves the addition of small amounts of oxygen to the wine in the months after fermentation (Parish et al., 2000). An increase in the contact of red wine with oxygen can contribute to a stabilization of color and to an improvement in the flavor and aroma (Pozo et al., 2010; Lopez-Cano et al., 2007). One of the effects of the treatment of micro could be the induction of changes in the structure of tannins that affect the changes that occur during aging, thus changing the perception of astringency of wine (González-Sanjosé et al., 2008; Llaudy et al., 2006). The long-term effects oxygenation on red wine have not yet been fully understood.

1.9 - INFLUENCE OF THE MATRIX WINE ON THE ASTRINGENCY

The interaction of the tannins of the wine with salivary proteins, and the size and stability of the complex protein-tannin, also depend on other characteristics of the wine matrix, in particular by the pH and ethanol content. Other factors may also have an impact on the perception of astringency, among which contents in organic acids, sugars, available as acetaldehyde, the viscosity, and the presence of other compounds that

interact with the tannins released as the proteins of the yeast and polysaccharides grapes. It was verified that the temperature at which wine is served has a minimum contribution on the sensation of astringency (Ross and Weller, 2008).

The ethanol content in red wine varies from 11% to 15% approximately. It has been shown that high contents reduce the perception of astringency (Fontoin et al., 2008; Vidal et al., 2004d) and change the sub-astringent wine (Demiglio and Pickering, 2008), although another study indicated an increase of astringency with increasing concentration of ethanol (Obreque-Slíer et al., 2010).

A decrease of astringency may, at least in part, be due to conformational changes of the tannins in the wines content increases in ethanol. This would be a reduction in the binding of tannins with proteins as well as self-association of tannins bound, limiting the formation of protein aggregates (Fontoin et al., 2008). It has been shown that an increase of the ethanol content between 10 and 20% destroys the hydrophobic interactions between the tannins and the cell wall material of the apple, in particular for compounds of high molecular weight with a high degree of galloylation (The Bourvellec et al., 2004). In addition, high concentrations of ethanol may also increase the smoothness of the oral cavity, reducing the perception of roughness (Demiglio and Pickering, 2008; Fontoin et al., 2008). An increase in the precipitation of the protein with a concentration of ethanol of 13% compared to aqueous solutions may be due to the variation of solubility of the complex protein-tannin formats (Obrque-Slíer et al., 2010).

The different level of tannins solubility in the wine may also affect the astringency results (Zanchi et al., 2007). Finally, an increase in viscosity of the solution with a higher content of ethanol could also reduce the perception of astringency as well as the protein-tannin (Demiglio and Pickering, 2008; Smith et al., 1996; Hagerman et al., 1998).

The pH of the wine generally varies from 3.2 to 3.8 and this difference is sufficient to cause change astrigency. It has been shown that lowering the pH of the wine and its solutions model increases the intensity of astringency between tannins and protein (Fontoin et al., 2008; Kallithraka et al., 1997c). This effect is more relevant for the increased concentration of individual organic acids such as the acid, malic acid, lactic acid and tartaric (Fontoin et al., 2008; Kallithraka et al. 1997c), although it has been shown that higher concentrations of organic acids along with high acidity

contribute to the characteristics "chalky" red wine (Gawel et al., 2007). It was also demonstrated that a combination of low pH and high concentration of organic acids is responsible for the increase of astringency in the sap of coconut fermented (Bags et al., 2006).

It is known that tannins bind to residual proteins or polysaccharides in the matrix of the wine, thus reducing the concentration available for the interaction with the salivary proteins and reducing the astringency (Escot et al., 2001). The reduction of astringency in the fruits in the ripening is attributed to an increase of the bond with the polysaccharide rather than to a decrease in the concentration of tannins (Luck et al., 1994; Taira et al., 1997). Polysaccharides different reduce the astrigency tannins with different mechanisms.

The gum arabic and the β-cyclodextrin preferentially bind polyphenols, inhibiting the interactions protein-tannin, while the properties of the polyelectrolyte pectin allow it to directly bind the complex of protein/polyphenols, in order to increase the water solubility of these complexes preventing them to precipitate in solution (Carvalho et al., 2006; McManus et al., 1985; Poncet-Legrand et al., 2007). The polysaccharides of the wine are ranked according to their net charge, which is neutral sour. The neutral polysaccharides in wine include arabinani and arabinogalactans. Acidic polysaccharides are pectins, including those of the berry cell walls and mannoproteins released by yeast during fermentation. The main polysaccharide acid wine is rhamnogalacturonan. All the polysaccharides reduce to some degree the perception of astringency, but among these polysaccharides acids have shown the best (Vidal et al., 2004b; Carvalho et al., 2006; Riou et al., 2002; Taira et al., 1997). It was also shown that the concentration of ethanol and ionic strength influence tannin-polysaccharides and the tannin-protein interactions (Poncet-Legrand et al., 2007).

High levels of sugar and anthocyanins in wine have been associated with a value lower astringency and a reduction of unpleasant feeling of "wrinkling" perceived in young wines (Gawel et al., 2007; Boselli et al., 2006; Sáenz- Navajas et al., 2010). The use of sweetening agents such as aspartame, however, has not had any effect on the astrigency perceived wine, suggesting that reported The association between high concentrations of sucrose and a reduced astringency may be due to the increased viscosity of the solution (Smith et al., 1996). The presence of agents that promote polymerization, such as acetaldehyde and glicossilic acid have shown a positive

influence on the perception of astringency, presumably due to increased molecular size of tannins (Vidal et al., 2004c). The dimers of flava-3-ols with ethyl bonds formed by reactions with acetaldehyde have been shown to have the same degree of astringency of dimers does not ethyl (Vidal et al., 2004c).

The interactions of these factors in the matrix wine, as well as differences in the structures and concentrations of tannins, affect the perception of astringency of red wines.

2. OBJECTIVES

Western Sicilian red wines are different from those of the centre north of the Italian peninsula and from those of the eastern part of the Region for the smooth taste and intense colour, if they come from grapes produced from grapes grown in order to achieve objectives high quality. It is said that the tannins of these grapes have reached a high level of maturity and that it is not necessary a maturation of the wine to refine them, to diminish their astringency and to stabilize the colour.

These assumptions that from the point of view of trade are trump cards, do not really have a scientific basis. Iin fact, neither the evolution of the structure and composition of tannins during the ripening of the grapes, or the influence of the environment in question on the synthesis of secondary metabolites quality (in this case polyphenols and aromas) or the nature of the tannins of the skins of native varieties (extension unit, report epicatechin/epigallocatechin and can galloylation), can galloylation tannins of the seeds.

The main objective of this research was the study of the evolution of skins proanthocyanidins during berry growth and ripening in different biotypes of two Sicilian cultivars grown in two different locations, trough the study of:

a) proanthocyanidins and flavanols reactive to vanillin;

b) determination of the constituent monomers, extension and terminals trough treatment of a condensed tannin with acid (depolymerization), in the presence of a nucleophile phloroglucinol (phloroglucynolisis) to calculate the mean degree of polymerization.

Some authors have applied these depolymerization methods to the wines. Especially, Drinkine *et al*. (2007) adapted the phloroglucinolysis to ethylidene bridged flavan-3-ols analysis which result from chemical modifications of flavan-3-ols occurring during wine making and aging. By this method, the authors showed that flavan-3-ol ethylidene bridges represented less than 4% of flavan-3-ol bonds and that the proportion of these linkages relative to native interflavan bonds increased with wine age. Nevertheless, Herderich and Smith (2005) underlined that this technique is limited for the characterization of wine tannins since the vast majority of wine tannins resists

the acid mediated depolymerization, allowing only a minor portion of tannin to be characterized. These depolymerization methods are difficult to implement and do not give information about the polymer distribution of a tannin fraction because all the polymers contained in the fraction are cleaved into monomer units in the course of the reaction. For example, when studied the depolymerization of a sample containing a mix of 50% of small tetrameric tannins and 50% larger octametric tannins, will yield an mDP of six, the same as considering a sample consisting of 100% hexameric tannins.

Therefore, about this process, a period of study-research in a Californian University (Fresno State University) was done in order to acquire other newer analytical methods for the characterization of tannins in wine.

3. MATERIALS AND METHODS

The grapes of Sicilian varieties were harvested in the 2013 and 2014 seasons in two different Sicilian experimental vineyards, located in Marsala (37°47'36.9"N 12°34'05.1"E) and Sambuca (37°40'47.2"N 13°04'38.9"E) districts. The plants were grafted on rootstock 1103 Paulsen, grown in vertical trellis system and with a Guyot pruning system. The soil of Marsala site is on a flat area, is clayey, deep, rich in organic matter and poor in skeleton; while, in Sambuca site, on hillside, the soil is clayey, rich in skeleton with a good organic matter content.

Biotypes studied, in the two experimental fields, were five as reported in tables 1 and 2 .

Agronomic surveys and samples of grapes were carried out during ripening, in three times: veraison, half-ripening (about 17 °Brix) and harvest. To follow the course of ripening, sugars concentration (°Brix), total acidity and pH were determined by Winescan (FOSS).

3.1 - AGRONOMIC SURVEYS

Meteorological data - Meteorological data were provided by the Servizio Informativo Agrometeorologico Siciliano (http://www.sias.regione.sicilia.it/). Weather stations are localized in Marsala (37°47'36.9"N 2°34'05.1"E) and Contessa Entellina (37°43'41.0"N 13°02'20.2"E). Over the development phase of grapes (from fruit set stage to harvest time) mean daily temperature, (T), mean daily rainfall and potential evapotranspiration (ET0) were recorded.

Leaf Relative Water Content - Relative water content (RWC) is the appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit. It estimates the current water content of the sampled leaf tissue relative the maximal water content it can hold at full turgidity. It is a measure of water deficit in the leaf. Normal values of RWC range between 98% in turgid and transpiring leaves to about 40% in severely desiccated and dying leaves.

In the two experimental fields for each biotype 20 leaves were collected and weighed (to obtain fresh leaf weight W), after which the samples is immediately

hydrated to full turgidity for 4h under normal room light and temperature. After 4 hours the samples were taken out of water and immediately weighed to obtain fully turgid weight (TW). Samples were then oven dried at 80 °C for 24h and weighed (after being cooled down in a desiccator) to determine dry weight (DW). All weighing is done to the nearest mg. Calculation:

$$\mathbf{RWC\ (\%) = [(W\text{-}DW) / (TW\text{-}DW)] \times 100,}$$

Where,

W – sample fresh weight

TW – sample turgid weight

DW – sample dry weight.

Pruning weight determination - Pruning weights for each biotype (3 plants to each genotype x environment) were obtained to determine vigour levels. All pruning weights are reported as kilograms on plant.

3.2 - SKINS SAMPLES PREPARATION

In order to analyse the skins tannins composition of grapes, gleanings from three separate plants (three repetitions) for each genotype x environment combination were collected. From gleanings, 25 berries were randomly collected and weighed. Chemical characteristics and the average berries weight of the grape samples are shown in 1 and 2 tables.

G x E combination			Date	Sampling	°Brix	pH	Total Acidity (g/L)	Berry weight (g)
Cultivar	Biotype	Site						
Nero d'Avola	A	Marsala						
			10/07/2013	I	5,00	2,57	32,63	159,24
			13/08/2013	II	17,70	2,92	13,00	155,40
			16/09/2013	III	25,17	3,27	9,54	143,24
Nero d'Avola	B	Marsala						
			10/07/2013	I	4,50	2,55	44,40	147,64
			13/08/2013	II	16,80	2,91	12,20	169,01
			16/09/2013	III	19,76	3,21	8,82	155,28
Nero d'Avola	C	Marsala						
			10/07/2013	I	5,50	2,58	42,68	194,13
			13/08/2013	II	18,20	2,96	12,00	190,55
			16/09/2013	III	16,02	3,15	8,87	190,40
Frappato	A	Marsala						
			10/07/2013	I	4,5	2,61	37,50	155,60
			16/09/2013	II	16,25	3,09	10,09	158,69
			01/10/2013	III	18,2	3,37	6,3	152,92
Frappato	B	Marsala						
			10/07/2013	I	4,50	2,53	41,55	148,65
			06/09/2013	II	17,92	3,04	9,62	154,89
			01/10/2013	III	18,57	3,12	8,88	144,09
Nero d'Avola	A	Sambuca						
			10/07/2013	I	5,75	2,64	37,65	162,07
			13/08/2013	II	19,00	3,32	11,74	158,84
			19/09/2013	III	32,30	3,71	7,81	148,47
Nero d'Avola	B	Sambuca						
			10/07/2013	I	4,75	2,58	39,30	105,27
			13/08/2013	II	18,80	3,28	11,65	85,72
			19/09/2013	III	20,95	3,33	7,19	127,67
Nero d'Avola	C	Sambuca						
			10/07/2013	I	5,00	2,55	40,28	181,88
			13/08/2013	II	18,60	3,43	10,82	178,88
			19/09/2013	III	26,00	3,42	8,70	177,08
Frappato	A	Sambuca						
			10/07/2013	I	5,25	2,51	41,78	156,00
			22/08/2013	II	17,80	2,89	10,70	148,31
			19/09/2013	III	26,00	3,42	8,70	156,40
Frappato	B	Sambuca						
			10/07/2013	I	5,25	2,47	45,05	104,61
			22/08/2013	II	18,60	2,98	9,50	120,60
			19/09/2013	III	22,89	3,19	9,33	126,04

Tab 1: 2013 harvest date, sugar (°Brix), pH and titrable acidity (g/L in tartarc acid) average berries weight of Genotype x Environment combinations

G x E combination			Date	Sampling	°Brix	pH	Total Acidity (g/L)	Berry weight (g)
Cultivar	Biotype	Site						
Nero d'Avola	A	Marsala						
			17/07/2014	I	4,8	2,58	39,8	126,83
			14/08/2014	II	17,4	2,97	10,2	210,04
			09/09/2014	III	23,4	3,45	7,2	206,09
Nero d'Avola	B	Marsala						
			17/07/2014	I	5,0	2,63	38,4	145,52
			25/08/2014	II	18,2	3,05	11,3	244,79
			09/09/2014	III	22,8	3,47	6,0	242,65
Nero d'Avola	C	Marsala						
			17/07/2014	I	4,5	2,55	41,4	151,99
			25/08/2014	II	18,8	2,88	11,5	247,09
			09/09/2014	III	21,0	3,47	7,8	261,45
Frappato	A	Marsala						
			17/07/2014	I	4,5	2,45	42,1	132,43
			09/09/2014	II	16,0	3,10	15,1	210,64
			21/09/2014	III	18,2	3,37	6,3	220,44
Frappato	B	Marsala						
			17/07/2014	I	4,8	2,45	37,5	137,53
			01/09/2014	II	16,2	3,15	13,9	215,71
			21/09/2014	III	20,6	3,10	9,9	211,99
Nero d'Avola	A	Sambuca						
			16/07/2014	I	6,3	2,58	36,7	113,41
			14/08/2014	II	17,5	2,94	15,8	197,92
			03/09/2014	III	26,4	3,21	8,4	224,43
Nero d'Avola	B	Sambuca						
			16/07/2014	I	6,3	2,60	35,6	80,56
			14/08/2014	II	17,2	2,97	15,2	132,96
			03/09/2014	III	23,6	3,19	8,4	158,84
Nero d'Avola	C	Sambuca						
			16/07/2014	I	5,8	2,60	40,6	122,15
			21/08/2014	II	17,5	3,01	18,2	211,85
			10/09/2014	III	22,8	3,27	7,1	215,48
Frappato	A	Sambuca						
			16/07/2014	I	5,8	2,55	37,0	107,81
			21/08/2014	II	17,2	2,84	14,2	178,03
			10/09/2014	III	22,2	3,05	6,0	180,53
Frappato	B	Sambuca						
			16/07/2014	I	5,5	2,59	34,5	97,08
			21/08/2014	II	17,8	3,01	10,3	169,39
			03/09/2014	III	23,2	3,26	6,6	170,92

Tab 2: 2014 harvest date, sugar (°Brix), pH and titrable acidity (g/L in tartarc acid) average berries weight of Genotype x Environment combinations

The skins of berries for each repetitions were splitted from the pulp and from the seeds. The skins were placed in 25 ml of tartaric buffer at pH = 3.2 (produced by adding the following 175 chemicals in the following order: 500 mL of distilled water, 5 g of tartaric acid, 22 mL 176 of 1N NaOH, 2 g of sodium metabisulphite and 120 mL ethanol 95%, the volume of 177 buffer was adjusted to 1 L by the addition of distilled water) (Di Stefano and Maggiorotto, 1995). The skins were placed in the buffer for four hours at room temperature. Then the extract was frozen.

After being thawed, skins were homogenized and centrifuged. The supernatant was collected in a 50 mL volumetric flask, the residue was washed again with tartaric buffer (pH 3.2) added to the volumetric flask and the volume was raised to 50 mL with tartaric buffer (pH 3.2).

Skins extracts were used to determination of:

- proanthocyanidins index (PI) and flavans reactive to vanillin (FRV) by spectrophotometric methods using a UV-vis spectrophotometer;
- Proanthocyanidins characterization after acid-catalyzed degradation in the presence of phloroglucinol by HPLC.

3.3 - WINEMAKING

The grapes from biotype considered in this study regards experimental vineyards harvested in September and vinified with the following standard procedure used in the "Valorizzazione dei Vitigni Autoctoni Siciliani" program: 80kg of grapes of each biotype were placed in plastic crates, moved to the winery, stored in a conditioned room for about 30 hours at 8°C, destemmed, crushed, placed in stainless steel tanks (50 L), added with 0,1 g/kg diammonium phosphate (DAP), 6mg/kg thiamine, and 0,2 g/kg reactivated dry yeast (OE Red 40, OE Italia, Marsala, Italy), and fermented at 26°C in a conditioned room. The musts were again added with 0.1 and 0,05g/L DAP and pumped over when reaching respectively 2 and 8 % alcohol. In addition, two punch downs were performed daily and the macerations were stopped at the end of alcoholic fermentation. Malolactic fermentation was induced by natural lactic acid bacteria. One winemaking was performed per accession. At the end of fermentations, the composition of the wines

was determined by means of a Winescan (FOSS) calibrated following EEC 2676 standard procedure (1990) for all parameters (results shown in tab. 3). Wines obtained were bottled and tasted from expert panel.

G x E combination			Ethanol (% vol.)	pH	Total Acidity (mg/L)
Cultivar	Biotype	Site			
Nero d'Avola	A	Marsala	13,140	2,830	9,590
Nero d'Avola	B	Marsala	13,640	2,990	7,990
Nero d'Avola	C	Marsala	11,660	2,960	8,840
Frappato	A	Marsala	10,020	3,380	7,080
Frappato	B	Marsala	11,220	3,350	7,430
Nero d'Avola	A	Sambuca	14,460	3,040	8,290
Nero d'Avola	B	Sambuca	12,850	3,120	6,860
Nero d'Avola	C	Sambuca	12,700	3,230	6,370
Frappato	A	Sambuca	12,300	3,360	6,050
Frappato	B	Sambuca	12,630	3,290	6,140

Tab. 3: chemicals characteristics of wine obtained in 2014 vintage

3.4 - ANALYSIS OF SKINS PROANTHOCYANIDINS

Proanthocyanidins index (Bates Smith Assay) - Skins extracts (0.2 mL) was placed in a 50 mL distillation flask in cold water. Ethanol 96% (12.3 mL) and HCl containing 300 mg/L of $FeSO4•7H2O$ (12.5 mL) were added to the flask, and the absorbance spectrum from 360 to 700 nm was recorded (E0). Next, the flask containing the solution was placed in boiling water. After 50 minutes, the absorbance spectrum from 360 to 700 nm was recorded again (E1). Proanthocyanidins index (PI) were calculated using the following equation:

$$\textbf{PI (mg/kg of berries)} = \textbf{(E1 - E0) x 1162.5 x (100/W)/0.2}$$

Where:

W was weight of 25 berries

Total Flavan-3-ol Content (Vanillin Assay) - Vanillin has the property of reacting with the 6 and 8 positions of the flavanols molecules, Therefore, using this colour

reaction is evaluated the contribution of flavanols having free these two positions (Di Stefano et al., 1989).

The results are expressed as (+)-Catechin, through the value of absorbance at 500 nm on 1 cm optical path. Skins extracts were diluted 10 times with methanol. Diluted skin extract (0,5 mL) was placed in a tube and added of 3 mL of a solution of vanillin to 4% in methanol. As white, in another test tube with dark glass was placed 0.5 mL of extract methanol and 3 mL of methanol. The two tubes were immersed in a bath of water and ice and after a few minutes, were added of 1.5 mL of concentrated HCl. After 15 minutes at room temperature, the absorbance E1 and E0 were read at 500 nm on a optical path of 1 cm. The results were calculated using the following equation:

FRV(mg/kg of berries) = A x d x (V/P)

Where:

A is:

- (277,26 x ΔE) - 3,58 to ΔE between 0,020 and 0,050
- (250 x ΔE) - 3,25 to ΔE between 0,050 and 0,180
- (314,23 x ΔE) - 3,25 to ΔE between 0,190 and 0,830

ΔE is the absorbance difference between white and sample with vanillin, **d** is the dilution factor (10 in this study) V is the extract volume (50 mL in this study) P is the weight of 25 berries.

Acid catalysis in presence of excess phloroglucinol (Phloroglucinolysis) - Phloroglucinolysis gives information on subunit composition, conversion yield, and mean degree of polymerization (mDP). Subunit composition of proanthocyanidins can be characterized by HPLC analysis. Treatment of a condensed tannin with acid, in the presence of a nucleophile such a thiol or less putrid and therefore much preferred phloroglucinol allows the subunit profile to be analysed by HPLC and the average molecular mass (expressed as “mean degree of polymerization, mDP”) to be calculated. Indeed, proanthocyanidins become depolymerized, releasing terminal subunits as flavan-3-ol monomers and extension subunits as electrophilic flavan-3-ol intermediates. The electrophilic intermediates can be trapped by the nucleophilic reagent to generate analyzable adducts. Most of our current knowledge about general composition and structure of grape and wine tannins has been obtained by depolymerization. Grape seeds

proanthocyanidins comprise only procyanidins (sub-units constituted of (+)-catechin and (−)-epicatechin) whereas grape skins proanthocyanidins include both procyanidins and prodelphinidins (sub-units constituted of (−)-epigallocatechin).

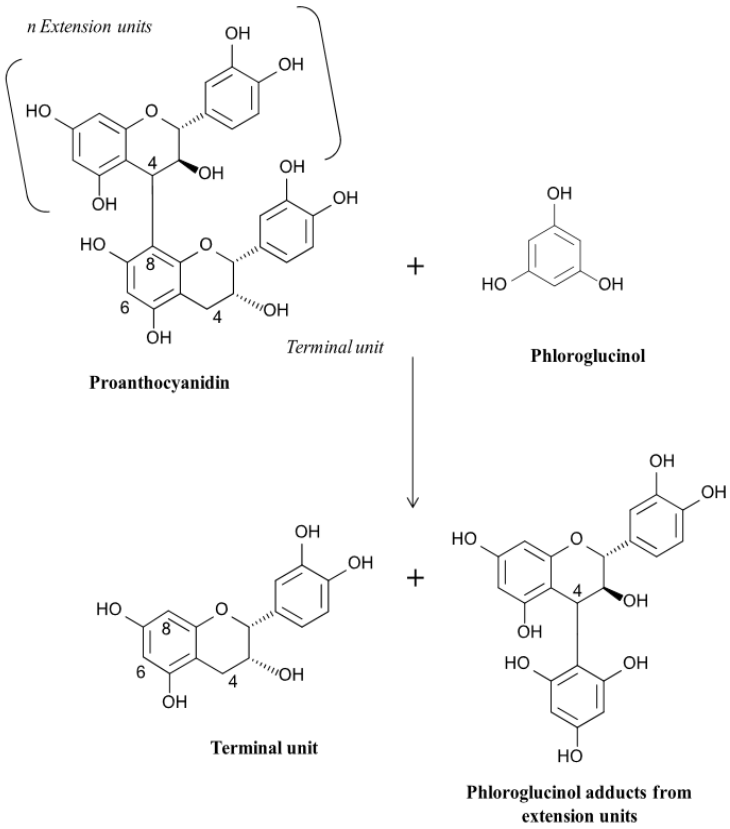


Figure. 6: proanthocyanidins – phloroglucynol reaction

Skin extract (5 ml) was acidified (0,01N H_2SO_4 15mL) an loaded into a Sep-Pak C18 cartridge preconditioned with methanol (2 mL) and 0,01N H_2SO_4 (3 mL). Cartridge was washed with 5 mL of 0.01N H_2SO_4, the monomeric catechins have been eliminated with 20 mL of ethyl ether (Sigma Aldrich). Oligomeric and polymer proanthocyanidins were recovered by elution with 10 mL of ethanol and collected in a distillation flask (50 mL).

Methanol extract was brought to dryness under vacuum. After evaporation, dried extracts were dissolved in 2 mL of MeOH containing 0.2N HCl, 100 mg of phloroglucinol, and 20 mg of ascorbic acid and heated for 25 min at 50 °C. Then, 4 ml of 100 mM sodium acetate solution were added to stop the reaction. Solution thus obtained was filtered and subjected to analysis by HPLC.

Released terminal subunits and extension subunit-phloroglucinol adducts were analysed by HPLC (Agilent), using a reversed-phase Grace C18 column (Econosphere C18 250 x 4.6 mm 5 um) protected by a guard column of the same material. The method utilized a binary gradient with mobile phases containing 1% v/v of H_3PO_4 10^{-3} M (solvent A), and acetonitrile (mobile phase B). The elution conditions were 0,5 mL/min at 40 °C; from 100 % A to 85 % A in 15 min, from 85 % A to 83 % A in 2 min, from 83 % A to 70 % A in 13 min, from 70 % A to 0 % A in 10 min, from 0% A to 100 % A in 10 min. Eluting peaks were monitored at 280 nm through DAD detector. Extension and terminals constituent monomers of proanthocyanidins were calculated in mg/kg, using a calibration curve (100, 500, 1000 mg/L of (+)-catechin).

The apparent average degree of polymerization (mDP) of proanthocyanidins, was determined dividing the content of the constitutive subunits for the content of terminal subunit.

3.5 - ANALYSIS OF WINES PROANTHOCYANIDINS

Wines analysis were conducted in Fresno State University (California). In particular, at the Department of Viticulture and Oenology, three types of chromatographic analyzes were performed: acid catalysis in presence of excess phloroglucinol (phloroglucinolysis*)*, gel permeation chromatography (GPC) and reversed-phase chromatography to determine thermodynamics interaction (stickiness, PRLP).

Samples preparation - 1ml of wine was loaded into a Sep-Pak C18 cartridge preconditioned with metanol (15 mL) and water (15 mL). Cartridge was washed with water (15 mL) to eliminate interfering molecules. Fixed polyphenols were elected with 9 ml of methanol and collected in a centrifuge tube. Methanol extract was brought to dryness with frees dryers (nome). After evaporation, dried extracts were dissolved in 1 mL of MeOH. Extracts thus obtained were used to phloroglucynolisis and molecular mass analysis.

Acid catalysis in presence of excess phloroglucinol (phloroglucinolysis) - The reversed-phase HPLC method used to analyze the proanthocyanidins following acid catalysis in the presence of excess phloroglucinol consisted of two Chromolith RP-18e

(10034.6 mm) columns connected in series and protected by a guard column (Purospher STAR RP-18e, 434 mm, 5 mm), all purchased from EM Science (Gibbstown, NJ, USA). The method utilized a binary gradient with water containing 1% (v/v) aqueous acetic acid (mobile phase A) and acetonitrile containing 1% (v/v) acetic acid (mobile phase B). Eluting peaks were monitored at 280 nm, and the elution conditions were as follows: column temperature 30 °C; 3.0 ml /min; 3% B for 4 min, a linear gradient from 3% to 18% B in 10 min, and 80% B for 2 min. The column was washed with 3% B for 2 min before the next injection. In order to estimate the quantitation of subunit products, the method of Kennedy and Jones was also utilized, using (−)-epicatechin as a standard.

Proanthocyanidins molecular mass - The 50% elution times for wine tannins were determined by GPC, using the method of Kennedy and Taylor (2003).

The high-performance GPC method used to analyse proanthocyanidins consisted of 2 PLgel (300 x 7.5 mm, 5 mm, 500 (effective molecular mass range of up to 4000 using polystyrene standards) by 1000 A° (effective molecular mass range of 500–30 000 using polystyrene standards) columns connected in series and protected by a guard column containing the same material (5037.5 mm, 5 mm), all purchased from Polymer Labs (Amherst, MA, USA). The sample injection amount was typically 20 µL. The isocratic method utilized a mobile phase consisting of *N*,*N*dimethylformamide containing 1% (v/v) glacial acetic acid, 5% (v/v) water and 0.15 *M* lithium chloride. The flow-rate was maintained at 1 ml /min with a column temperature of 60 °C and elution monitored at 280 nm. Calibration curves were constructed using fractionated proanthocyanidins, by correlating their average molecular mass (determined by acid catalysis) with their cumulative mass distribution at 50%.

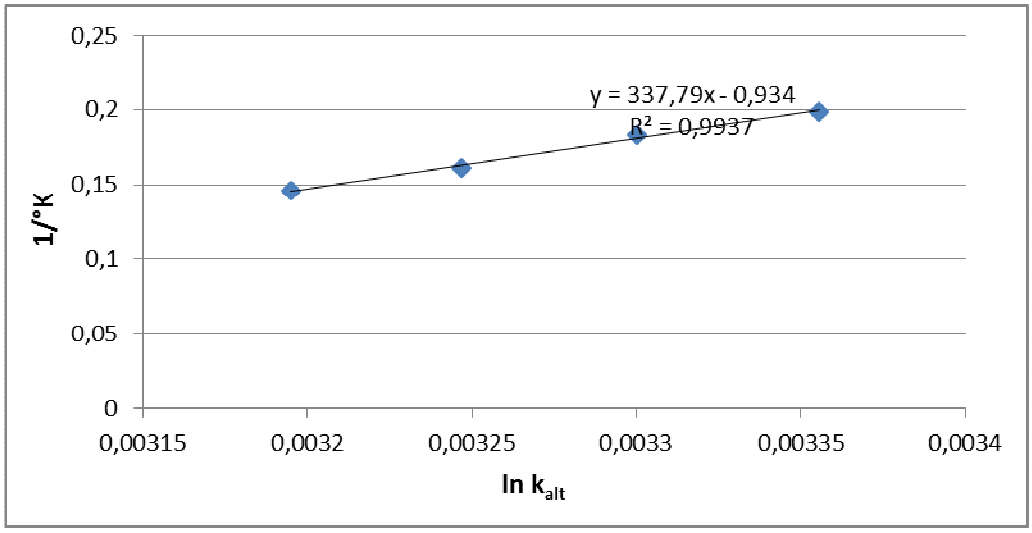
Proanthocyanidins stickiness - The HPLC method for measuring the thermodynamics of tannin interaction (for measuring tannin stickiness) utilized a rigid spherical polystyrene divinylbenzene reversed-phase column (PLRP-S, 2.1 × 50 mm, 100 Å, 3 µm, Agilent Technologies) protected with a guard column (PRP-1, 3 × 8 mm, Hamilton Co., Reno, NV, USA). Tannin isolates (5 mg/mL) were dissolved in an aqueous solution containing methanol (15% v/v), 40 mM sodium acetate, and 20 mM HCl.

Prepared tannin-containing solutions were filtered using a 0.45 µm PTFE syringe filter (Grace Davison Discovery Scientific, Deerfield, IL, USA) prior to injection (35 µg). The mobile phases were 1.5% (w/w) H_3PO_4 in water (180 mM, mobile phase A)

and 20% (v/v) mobile phase A in acetonitrile (B) with a flow rate of 0.21 mL/min. The linear gradient was as follows (time in min (%B)): 0 (14%), 18.0 (34%), 18.0−19.0 (34%), 21.5 (70%), 21.5−24.0 (70%), and 24.0−28.0 (14%). To determine tannin stickiness, samples were run at four column temperatures (25−40 °C, in 5 °C increments). A blank sample (water) was run at all temperatures and was subtracted from sample signals to eliminate background absorbance. All temperatures were converted into kelvin to calculate thermodynamic parameters in SI units. Tannin elution was monitored at 280 nm. Chromatograms were integrated. Briefly and following baseline subtraction of a water blank, a baseline at 0 mAU across the sample peak area was performed. Following this, the resulting peak area was split at 16.8 min, related to the elution of tanninP. The tannin eluting prior to 16.8 min was separated from overlapping resolved material by tangent skimming. From integration results, the alternative retention factor was calculated as follows:

Kalt= tannin(t)/tannin(t) – tannin(p)

where, tannin(t) is the total tannin peak area and tannin (p) is the partial tannin peak area. The specific enthalpy was calculated by plotting the natural logarithm (ln) of k_{alt} versus the reciprocal of the column temperature in Kelvin degree at each of the four temperatures. The specific enthalpy of interaction (tannin stickiness) was calculated from the slope of the best fit line (slope equivalent to −ΔH°/R).



The concentration of tannin (on the basis of tannin(t)) was reported in (−)-epicatechin equivalents using an (−)-epicatechin standard and was determined at 303 K (30 °C). In addition to (−)-epicatechin, a grape skin or wine tannin standard (mDP = 39.0 and 15.4, respectively) served as a stickiness standard. Stickiness standards were prepared by dissolving grape skin or wine tannins in an aqueous solution containing methanol (15%, v/v), 40 mM sodium acetate, and 20 mM HCl.

4. RESULTS AND DISCUSSION

Along the grapes ripening of Nero d'Avola and Frappato in the two different experimental vineyards, in Marsala and Sambuca, the following data have been collected:

a) proanthocyanidins and flavans reactive to vanillin in skins (vintage 2013 and 2014);

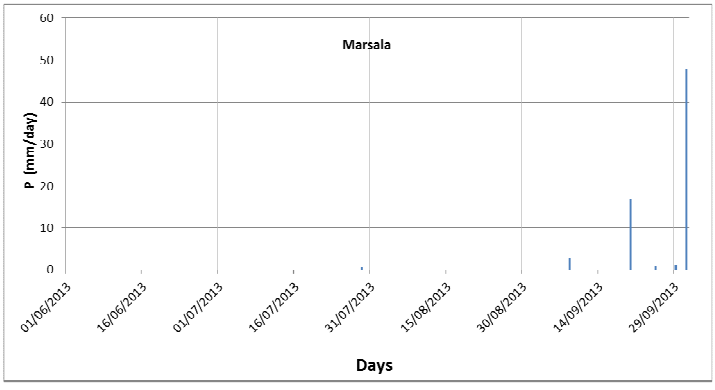
b) incorporation of the monomers, of extension and terminals, and the average degree of polymerization of proanthocyanidins after acid hydrolysis in the presence of an excess of phloroglucinol (vintage 2014);

A research period in California (University of Fresno) to acquire information on the characteristics of the wines tannins obtained from experimental microvinifications of grapes (vintage 2014).

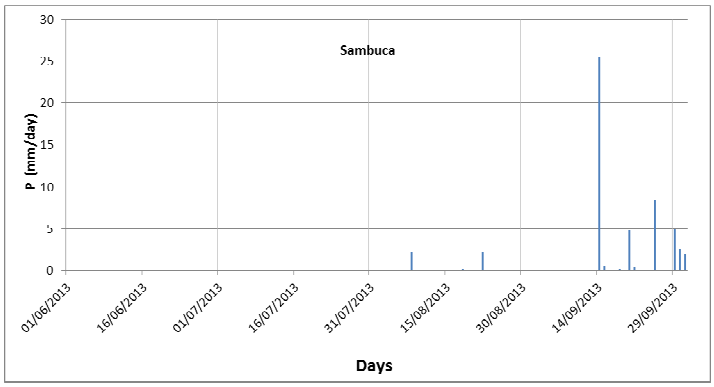
4.1 - AGRONOMIC SURVEYS

Meteorological data - 2013: in both experimental sites (graph. 1 and 2), there weren't rainfall events in the weeks between July and mid-August. During the weeks between mid-August and September, in Marsala occurred a few days of intense rain; while, in Sambuca site many rainy days, but not very intensive, were recorded.

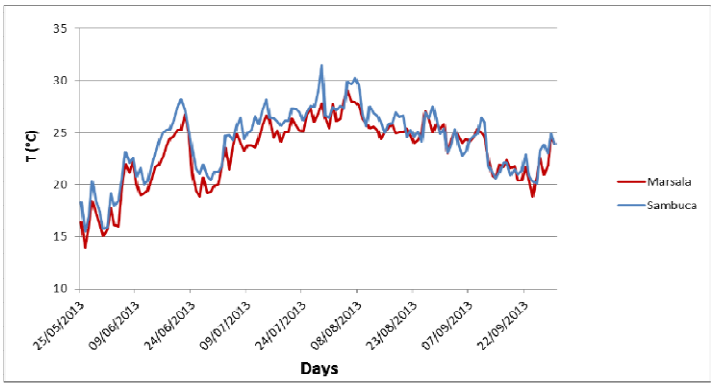
The average daily temperature (graph. 3) as well as the potential evapotranspiration (graph. 4), was higher in Sambuca site than Marsala site. This difference was observed especially in the weeks between July and August; later, the differences were reduced probably due to the rainy weather.



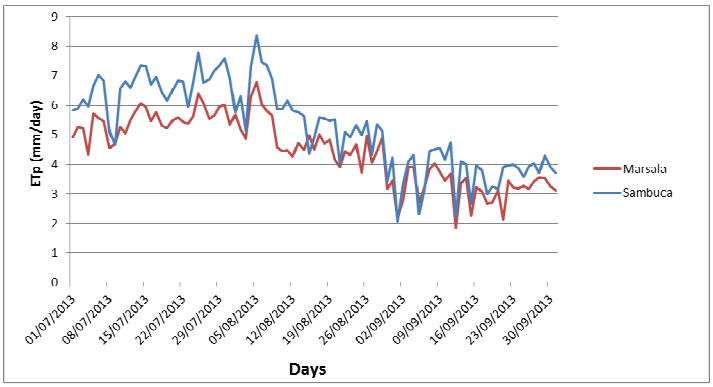
Graphic 1: Daily total precipitation (P) detected during ripening in Marsala site (2013)



Graphic 2: Daily total precipitation (P) detected during ripening in Marsala site (2013)

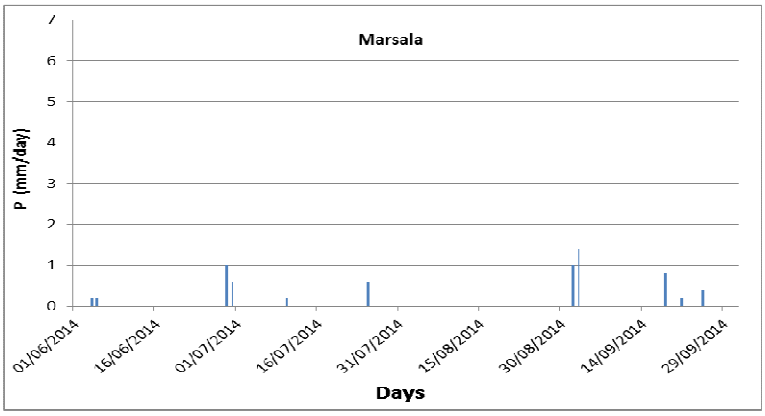


Graphic 3: daily temperature (T) detected during ripening in Marsala and Sambuca sites (2013)

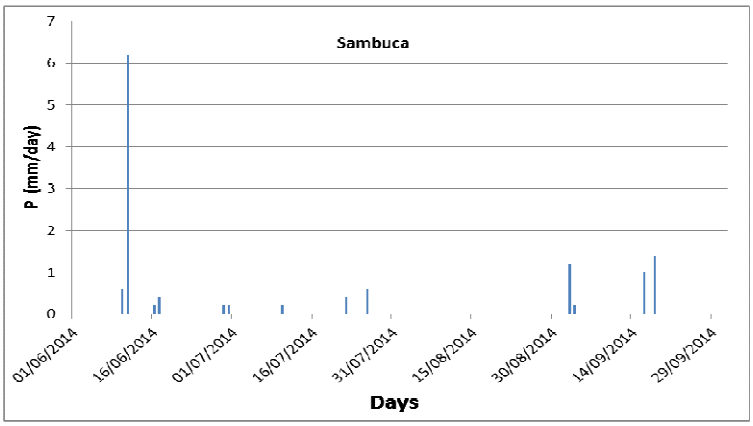


Graphic 4: daily potential evapotranspiration (ETp) detected during ripening in Marsala and Sambuca sites (2013)

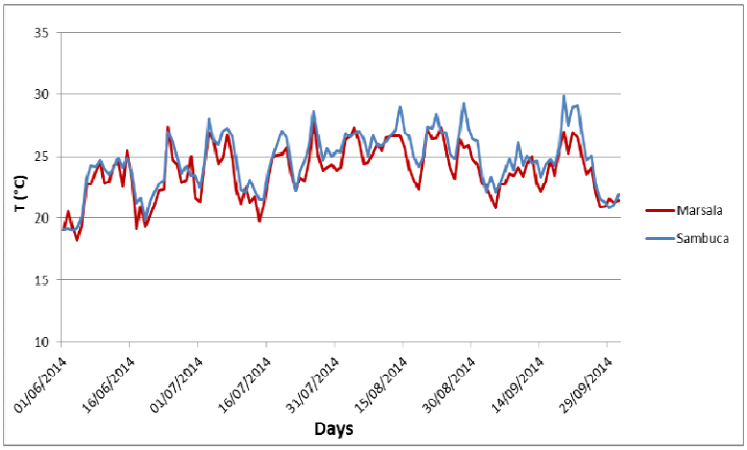
2014: in the two experimental sites, over the ripening, there weren't major rainfall events (graph 5 and 6). Compared to Marsala, the average daily temperature (graph. 7) was higher in Sambuca with differences which have widened in the last weeks of August and September. The daily evapotranspiration (ETp) was higher in Sambuca than Marsala during all the weeks corresponding to the period of ripening of the grapes (graph. 8).



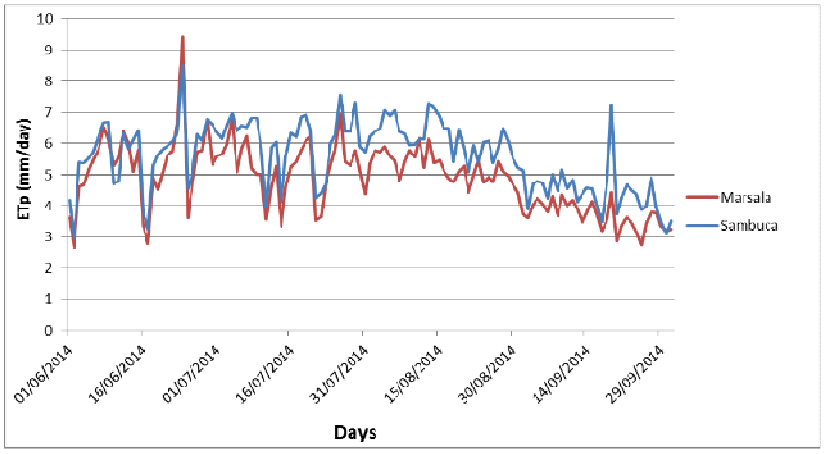
Graphic 5: Daily total precipitation (P) detected during the ripening in the Marsala site (2014)



Graphic 6: Daily total precipitation (P) detected during the ripening of the grapes in the Sambuca site (2014)



Graphic 7: daily temperature (T) detected during ripening in Marsala and Sambuca sites (2014)



Graphic 8: daily potential evapotranspiration (ETp) detected during ripening in Marsala and Sambuca sites (2014)

Leaf relative water content (RWC) - 2013: during ripening of grapes, leaf RWC levels were high in both experimental sites (tab. 3). This, may be, due to the fact that the soils were supplied with water from rainy summer events previously described.

G x E combination		I sampling			II sampling			III sampling		
Cultivar	Biotype	Marsala	Sambuca	**Average**	Marsala	Sambuca	**Average**	Marsala	Sambuca	**Average**
Nero d'Avo	*A*	92,30	91,56	**91,93**	94,48	95,21	**94,85**	95,22	97,08	**96,15**
Nero d'Avo	*B*	94,80	92,99	**93,89**	92,31	93,42	**92,86**	97,80	93,74	**95,77**
Nero d'Avo	*C*	93,99	96,85	**95,42**	89,87	95,00	**92,44**	96,85	95,89	**96,37**
Frappato	*A*	92,17	80,14	**86,15**	94,21	89,08	**91,64**	95,23	98,29	**96,76**
Frappato	*B*	95,68	95,40	**95,54**	95,68	92,60	**94,14**	97,81	97,66	**97,73**
Average		**93,79**	**91,39**		**93,31**	**93,06**		**96,58**	**96,53**	

Table 3: leaf relative water content (2013 vintage)

2014: the average leaf relative water content registered in two experimental sites shows that water levels in Marsala remained lower than in Sambuca, during the course of ripening (tab. 4). In Marsala, RWC passed from 94.74% (first sampling) to 84.76% (harvest time); while, in Sambuca, from 95.02% to 87.60%. At biotypes levels, in all cases the, the leaf relative water content were indicated a moderate water stress in the harvest time, especially for the B biotype of Nero d'Avola that registered a RWC of 84.12 %.

G x E combination		I sampling			II sampling			III sampling		
Cultivar	Biotype	Marsala	Sambuca	**Average**	Marsala	Sambuca	**Average**	Marsala	Sambuca	**Average**
Nero d'Avo	*A*	94,24	94,79	**94,51**	96,25	90,26	**93,25**	87,77	85,20	86,48
Nero d'Avo	*B*	96,80	95,97	**96,38**	96,16	96,07	**96,12**	85,92	82,33	84,12
Nero d'Avo	*C*	96,62	94,97	**95,79**	91,75	93,55	**92,65**	87,00	86,53	86,76
Frappato	*A*	94,61	94,70	**94,66**	91,96	95,05	**93,50**	83,78	88,89	86,34
Frappato	*B*	91,42	94,69	**93,05**	80,62	96,17	**88,40**	79,32	95,02	87,17
Average		**94,74**	**95,02**		**91,35**	**94,22**		**84,76**	**87,60**	

Table 4: leaf relative water content (2014 vintage)

Pruning weight - 2013: biotypes in Marsala site (0.91 kg/plant) were found to be much more vigorous than biotypes in Sambuca site (0.84 kg/plant) with the exception of the B biotype of Frappato cultivars. A biotype of Nero d'Avola cultivars (0.99 kg/plant) and the both biotypes of Frappato (A = 0.96 and B= 0.94 kg/plant) showed a higher pruning wood weight than other biotypes; While the B biotype of Nero d'Avola (0.73 kg/plant) was less vigorous in both experimental sites (tab. 5).

G x E combination		Marsala (kg/plant)	Sambuca (kg/plant)	**Average**
Cultivar	Biotype			
Nero d'Avola	*A*	1,01	0,98	**0,99**
Nero d'Avola	*B*	0,77	0,70	**0,73**
Nero d'Avola	*C*	0,91	0,61	**0,76**
Frappato	*A*	1,01	0,91	**0,96**
Frappato	*B*	0,87	1,00	**0,94**
Average		**0,91**	**0,84**	

Table 5: pruning weight (2013 vintage)

2014: the average weight of the pruning wood (tab. 6) found in the vineyards of Sambuca site (1.80 kg/plant) was higher than Marsala (1,08 kg/plant). The biotypes which showed greater vegetative expression were those of Frappato cultivars (A =1.64, B = 1.71 kg/plant). As 2013, the B biotype of Nero d'Avola was less vigorous in both experimental sites (kg/plant 1.06).

G x E combination		Marsala	Sambuca	**Average**
Cultivar	Biotype	(kg/plant)	(kg/plant)	
Nero d'Avola	*A*	1,09	1,83	**1,46**
Nero d'Avola	*B*	0,90	1,22	**1,06**
Nero d'Avola	*C*	1,16	1,48	**1,32**
Frappato	*A*	1,16	2,11	**1,64**
Frappato	*B*	1,07	2,35	**1,71**
Average		**1,08**	**1,80**	

Table 6: pruning weight (2014 vintage)

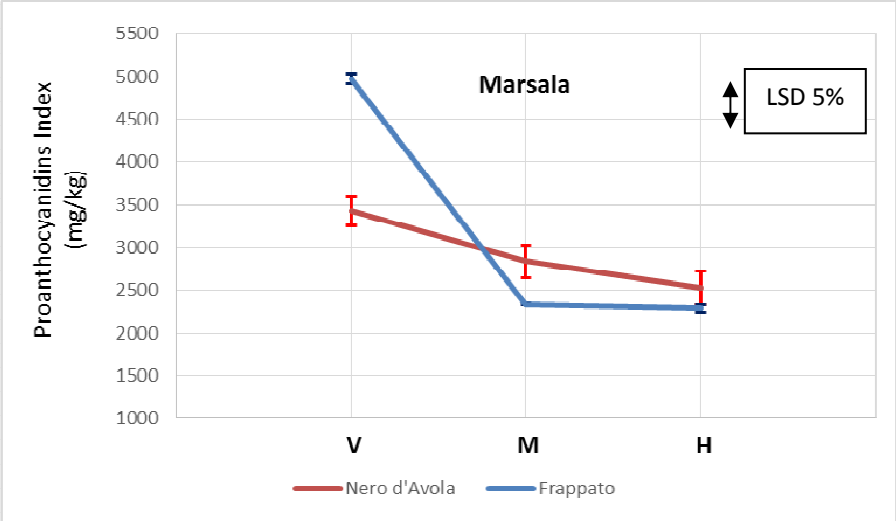
4.2 - EVALUATION OF SKINS PROANTHOCYANIDINS AND FLAVANS REACTIVE TO VANILLIN OF THE GRAPES DURING RIPENING

Over the two years experiences (2013 and 2014), as reported on the tables regarding the skins of Nero d'Avola and Frappato, mean levels of Proanthocyanidins (PI), flavans reactive to vanilli (FRV) removable from skins with tartaric buffer (pH3,2) decreased during the course of ripening compared to values recorded at the first sampling (pre-veraison). The degree of polymerization, deduced from the relationship between FRV/PI increased between veraisona and harvest time. Observed trends confirmed that both proanthocyanidins and flavans reactive to vanillin were synthesized prior to veraison; then during maturation, they suffered a decrease. This decrease can be caused from the diversion of intermediary metabolites (cyanidin and delphinidin) towards the synthesis of anthocyanins or more or less known phenomena of oxidation and transformation of proanthocyanidins. In the latter case, we could not exclude the progress of flavanols that, however, would have been exceeded by oxidative transformations or degradation.

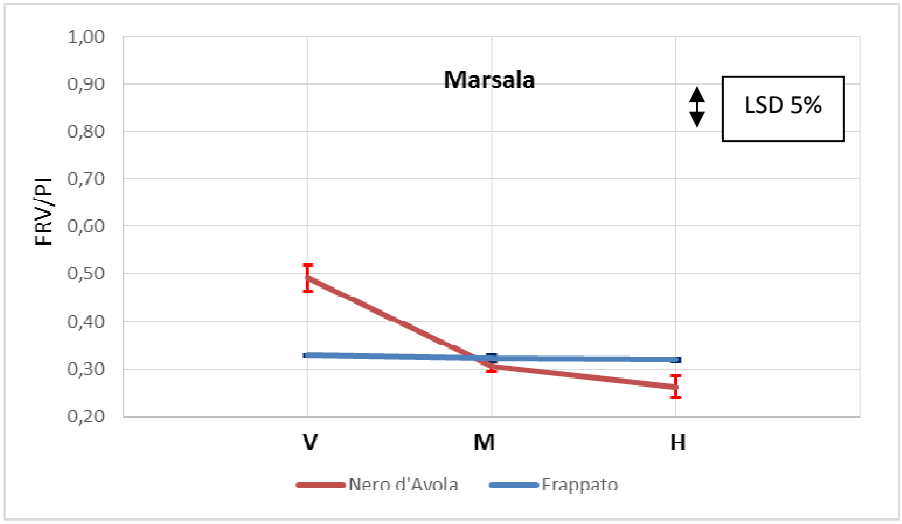
2013: *cultivar x site interaction* - the data show a concentration of proanthocyanidins, flavans reactive to vanillin and a degree of polymerization tend were high Marsala experimental vineyard compared to Sambuca site (tab. 7). Anomalies in the results corresponding to cultivar Frappato at Sambuca are evident.

G x E combination		Sampling	Proanthocyanidins index (PI)		Flavans reactve to vanillin (FRV)		FRV/PI	
Cultivar	Site		mg/kg	Std. Err.	mg/kg	Std. Err.	value	Std. Err.
Nero d'Avo	Marsala							
		I	3433,59	166,26	1680,27	101,33	0,49	0,03
		II	2838,25	191,70	873,11	81,99	0,31	0,01
		III	2528,93	194,43	634,48	24,34	0,26	0,02
Frappato	Marsala							
		I	4972,70	60,07	1486,99	52,83	0,33	0,00
		II	2338,88	6,64	755,12	14,45	0,32	0,01
		III	2292,62	43,84	732,54	20,07	0,32	0,00
Nero d'Avo	Sambuca							
		I	2331,50	497,72	850,27	183,57	0,36	0,01
		II	2098,07	335,24	609,15	142,88	0,27	0,02
		III	2023,75	352,35	542,43	97,25	0,26	0,01
Frappato	Sambuca							
		I	1739,08	5,12	1486,88	172,67	0,85	0,11
		II	2284,07	495,54	1005,95	152,93	0,56	0,19
		III	1428,43	52,10	828,34	265,33	0,60	0,21

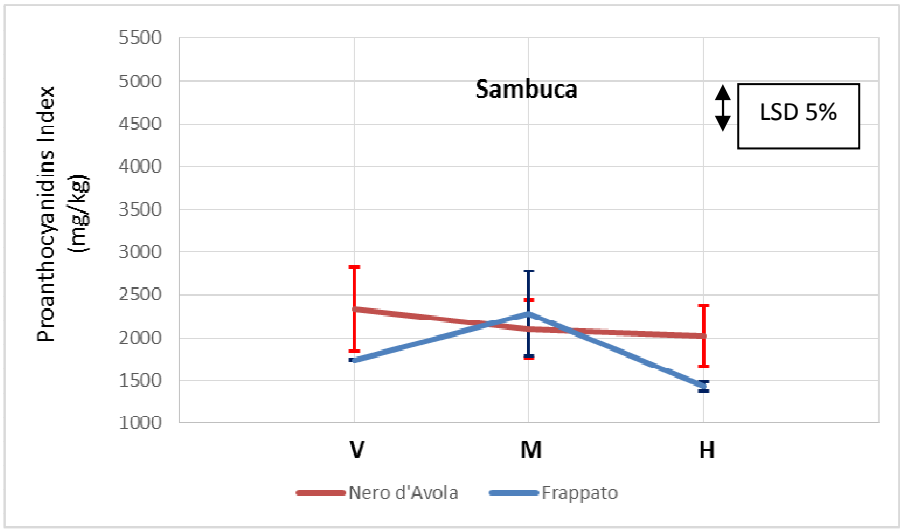
Table 7: spectrophotometric analysis recorded from skins extracts during ripening in each cultivar (2013 vintage)



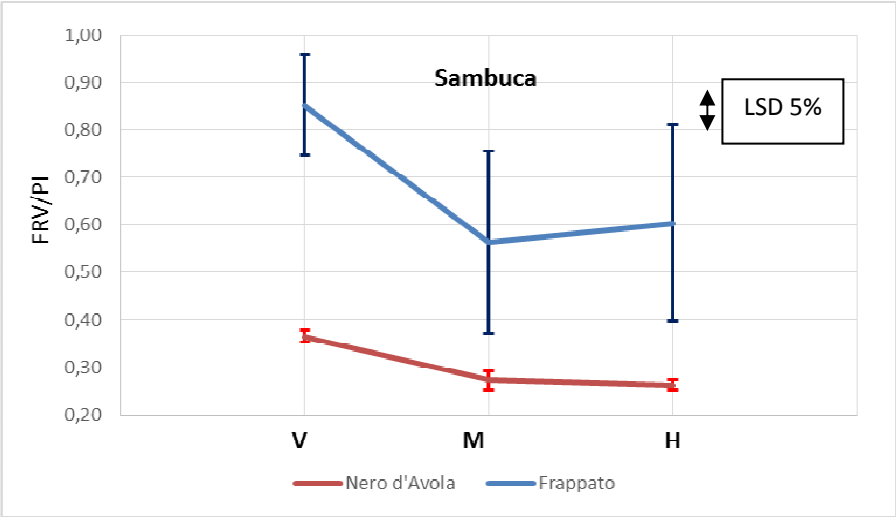
Graphic 9: evolution of skins proanthocyanidins of Nero d'Avola and Frappato in Marsala site (2013 vintage)



Graphic 10: evolution of FRV/PI ration of skins proanthocyanidins of Nero d'Avola and Frappato in Marsala site (2013 vintage)



Graphic 11: evolution of skins proanthocyanidins of Nero d'Avola and Frappato in Sambuca site (2013 vintage)



Graphic 12: evolution of FRV/PI ratio of skins proanthocyanidins of Nero d'Avola and Frappato in Marsala site (2013 vintage)

Biotype x site interaction - At harvest, significant differences were found between the various biotypes in two different experimental fields (tab. 8).

For the Nero d'Avola cultivar, in Marsala site, total proanthocyanidins and their degree of polymerization were higher in the biotype A (PI = 3185.17 mg/kg, FRV/PI = 0.21) compared to B and C ; while in Sambuca B biotype (PI = 2011.15 mg/kg, FRV/PI = 0.25) had the highest content of proanthocyanidins compared to biotypes A and C and with a degree of polymerization tends to be equal to the biotype C but greater biotype A.

In Frappato cultivar, B biotype in Marsala (PI = 2368.55 mg/kg) showed a proanthocyanidins content higher than biotype A; FRV/PI ratio shows that B biotype was higher proanthocyanidins polymerisation than A biotype. Even in Sambuca, B biotype (PI = 1518,67 mg/kg) basically showed a higher concentration of proanthocyanidins compared with A with a degree of polymerization of 0.24, no-comparable with value, value (FRV/PI = 0.96), corresponding to A biotype (probably error).

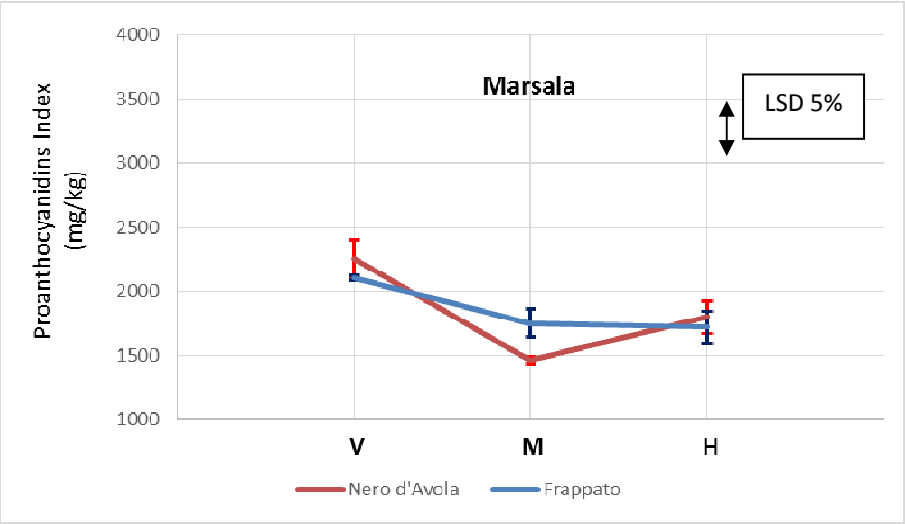
G x E combination			Proanthocyanidins index (PI)		Flavans reactve to vanillin (FRV)		FRV/PI	
Cultivar	Biotype	Site	mg/kg	Std. Err.	mg/kg	Std. Err.	value	Std. Err.
Nero d'Avo	*A*	Marsala	3185,17	221,70	661,90	62,90	0,21	0,01
Nero d'Avo	*B*	Marsala	2069,56	589,48	689,81	173,99	0,34	0,02
Nero d'Avo	*C*	Marsala	2332,05	133,38	551,73	53,87	0,24	0,04
Frappato	*A*	Marsala	2216,69	194,38	697,78	65,55	0,31	0,01
Frappato	*B*	Marsala	2368,55	35,41	767,29	59,63	0,32	0,02
Nero d'Avo	*A*	Sambuca	2305,82	221,35	688,54	98,92	0,28	0,03
Nero d'Avo	*B*	Sambuca	2911,16	389,71	732,24	146,31	0,25	0,03
Nero d'Avo	*C*	Sambuca	854,28	58,63	206,50	11,50	0,24	0,01
Frappato	*A*	Sambuca	1338,19	60,58	1287,90	51,04	0,96	0,03
Frappato	*B*	Sambuca	1518,67	83,08	368,78	12,59	0,24	0,01

Table 8: spectrophotometric analysis recorded from skins extracts during ripening in each biotype (2013 vintage)

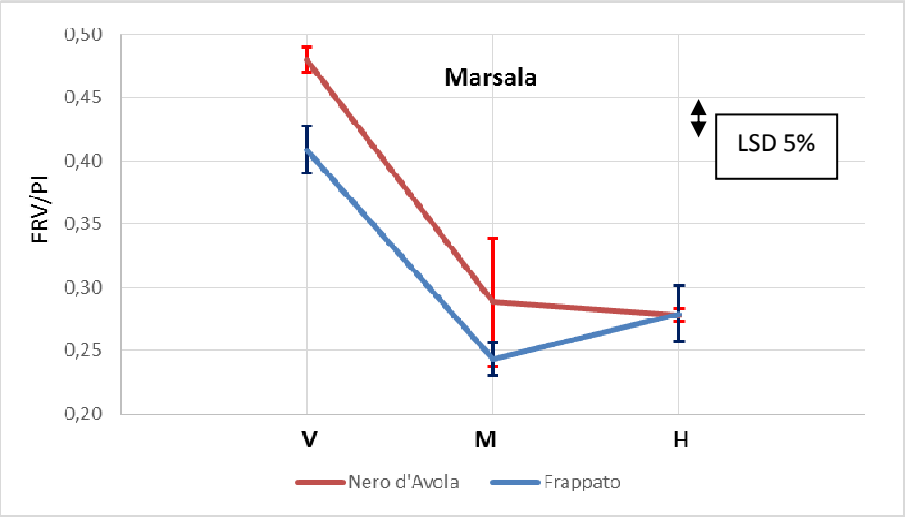
2014: *cultivar x site interaction* - the data show that proanthocyanidins concentration, reactive flavans reactive to vanillin and degree of polymerization were higher in Sambuca experimental vineyard than Marsala (tab. 9). In Sambuca site, during ripening, both proanthocyanidins and both flavans reactive to vanillin, appeared to have a more intense decrease than Marsala.

G x E combination		Sampling	Proanthocyanidins index (PI)		Flavans reactve to vanillin (FRV)		FRV/PI	
Cultivar	Site		mg/kg	Std. Err.	mg/kg	Std. Err.	value	Std. Err.
Nero d'Avola	Marsala							
		I	2253,57	151,08	1068,59	59,00	0,48	0,01
		II	1463,45	28,78	409,10	3,78	0,29	0,05
		III	1798,79	124,99	493,34	23,87	0,28	0,01
Frappato	Marsala							
		I	2104,45	15,81	868,58	45,11	0,41	0,02
		II	1753,58	106,48	444,46	27,43	0,24	0,01
		III	1721,01	128,44	451,21	11,40	0,28	0,02
Nero d'Avola	Sambuca							
		I	2771,16	504,66	1297,37	247,48	0,46	0,01
		II	2086,61	263,83	626,42	190,76	0,26	0,05
		III	1613,69	122,45	412,46	70,97	0,24	0,02
Frappato	Sambuca							
		I	3478,93	187,62	1582,18	95,78	0,46	0,01
		II	1812,45	74,51	562,07	18,28	0,31	0,01
		III	1877,44	132,14	523,46	37,00	0,28	0,01

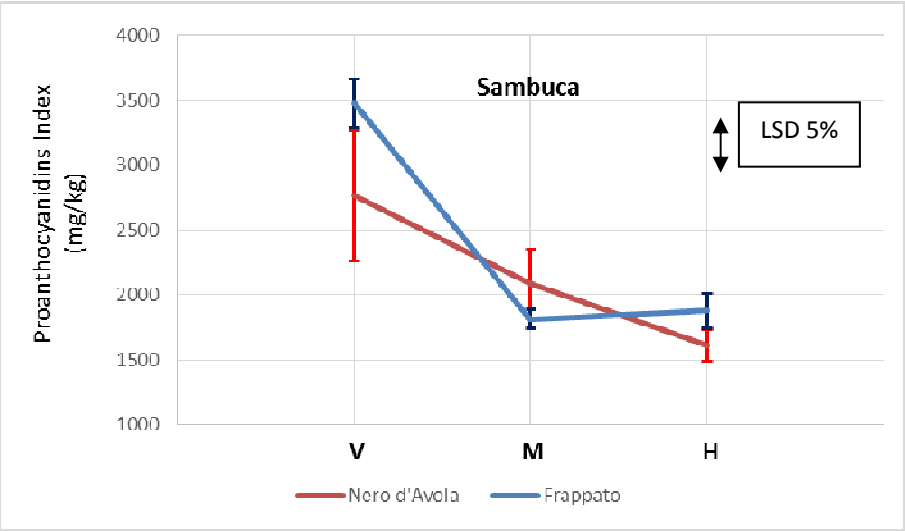
Table 9: spectrophotometric analysis recorded from skins extracts during ripening in each cultivar (2014 vintage)



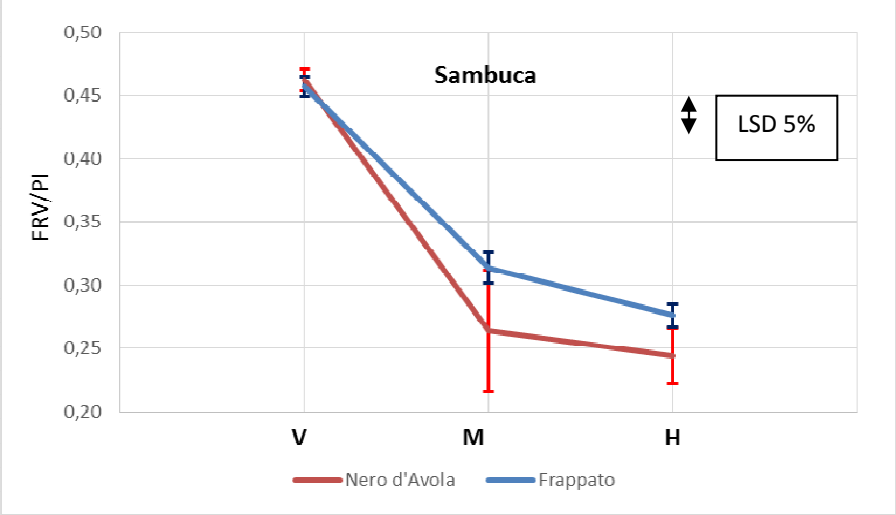
Graphic 13: evolution of skins proanthocyanidins of Nero d'Avola and Frappato in Marsala site (2014 vintage)



Graphic 14: evolution of FRV/PI ratio of skins proanthocyanidins of Nero d'Avola and Frappato in Marsala site (2014 vintage)



Graphic 15: evolution of skins proanthocyanidins of Nero d'Avola and Frappato in Sambuca site (2014 vintage)



Graphic 16: evolution of FRV/PI ratio of skins proanthocyanidins of Nero d'Avola and Frappato in Sambuca site (2014 vintage)

Biotype x site interactions - at harvest (tab. 10), in Marsala site ,A and B biotypes of Nero d'Avola (respectively PI = 1885,4 and 2122,87 and mg/kg), showed a higher proanthocyanidins concentration than C biotype, and between them there weren't differences in terms of degree of polymerization. In Sambuca site B biotype of Nero d'Avola (PI = 2037.01 mg/kg, FRV/PI = 0.32) prevailed on A and C biotypes both for the proanthocyanidins concentration, and for the their degree of polymerization.

In Frappato cultivar, in Marsala, B biotype (PI = 2108,79 mg/kg, FRV/PI = 0.24) prevailed on A biotype for the proanthocyanidins concentration and as tendency even for the degree of polymerization. In Sambuca, between the two biotypes of Frappato there weren't important differences in the proanthocyanidins concentration and their degree of polymerization.

.

G x E combination			Proanthocyanidins index (PI)		Flavans reactve to vanillin (FRV)		FRV/PI	
Cultivar	Biotype	Site	mg/kg	Std. Err.	mg/kg	Std. Err.	value	Std. Err.
Nero d'Avola	*A*	Marsala	1885,42	92,00	532,93	24,01	0,28	0,00
Nero d'Avola	*B*	Marsala	2122,87	203,59	536,41	26,06	0,26	0,04
Nero d'Avola	*C*	Marsala	1388,08	150,98	410,68	105,55	0,29	0,06
Frappato	*A*	Marsala	1333,24	305,81	429,31	151,13	0,32	0,03
Frappato	*B*	Marsala	2108,79	493,37	473,10	60,29	0,24	0,05
Nero d'Avola	*A*	Sambuca	1424,36	82,60	271,58	6,14	0,19	0,01
Nero d'Avola	*B*	Sambuca	2037,10	164,06	657,39	118,06	0,32	0,03
Nero d'Avola	*C*	Sambuca	1379,62	37,47	308,42	18,96	0,22	0,01
Frappato	*A*	Sambuca	2163,06	405,06	587,56	183,14	0,26	0,03
Frappato	*B*	Sambuca	1591,83	90,53	459,37	56,99	0,29	0,03

Table 10: spectrophotometric analysis recorded from skins extracts during ripening in each biotype (2014 vintage)

4.3 – EVALUATION OF MONOMERS CONSTITUENT, EXTENSION AND TERMINALS, AND THE DEGREE OF POLYMERIZATION OF PROANTHOCYANIDINS DURING THE RIPENING OF THE GRAPES (CHROMATOGRAPHIC DETERMINATIONS)

The acid catalyzed hydrolysis of the proanthocyanidins extracts in the presence of a large excess of phloroglucinol of Nero d'Avola and Frappato skins, highlighted the (+) - catechin as a terminal unit and the (-) - epicatechin, (-) - epigallocatechin and (-) - epicatechin-3-0-gallate, as phloroglucinol – linked, determined as extension unit.

The same terminal units and extension have been identified in skins of Chardonnay from Kennedy and Jones (2001).

Subunit composition of extension in harvest time - the two experimental sites, the proanthocyanidins composition of different cultivar an biotypes, in percentage terms, showed no significant differences (tab. 11). The epicatechin was the main monomer of skins proanthocyanidins of all biotypes. However, Frappato cultivar showed a higher concentration of epigallocatechin than Nero d'Avola biotypes. It is possible that Frappato synthesize more epigallocatechin than Nero d'Avola due to genetic factors. In Marsala, A and B biotypes of Frappato had a content of epigallocatechin, as extension unit, respectively, equal to 11.23 and 6.76%; while, in Sambuca, for the same biotypes were recorded values respectively equal to 8.53 and 9.94%.

G x E combination			EGC-P	C-P	EC-P	ECG-P
Cultivar	Biotype	Site	%	%	%	%
Nero d'Avola	*A*	Marsala	5,55	2,57	88,41	3,48
Nero d'Avola	*B*	Marsala	2,85	1,77	94,83	0,55
Nero d'Avola	*C*	Marsala	6,93	0,15	85,81	7,11
Frappato	*A*	Marsala	11,23	2,85	82,94	2,98
Frappato	*B*	Marsala	6,76	3,55	87,93	1,77
Nero d'Avola	*A*	Sambuca	7,03	3,95	84,14	4,89
Nero d'Avola	*B*	Sambuca	6,99	5,16	84,81	3,03
Nero d'Avola	*C*	Sambuca	5,87	5,26	82,08	6,78
Frappato	*A*	Sambuca	8,53	6,12	82,24	3,11
Frappato	*B*	Sambuca	9,94	2,85	83,81	3,40

Table 11: proanthocyanidins extentions subunits composition

Cultivars x site interactions - In Marsala site, the total subunits concentration of Nero d'Avola cultivar decreased from 461,89 (first sampling) to 310,73 mg/kg (harvest time), while Frappato cultivar switched from 244,97 to 196,97 mg/kg.

In Sambuca, a higher decrease than in Marsala were recorded. In fact, for Nero d'Avola, the total concentration of subunit decreased from 552,11 (first sampling) to 348,23 mg/kg (harvest time) and Frappato from 555,25 to 293,68 mg/kg.

During ripening, both extension subunits, both terminals subunits have undergone a progressive decrease (tab. 12)

According to Kennedy et al. (2000b), flavonoids monomers and monomers constituting proanthocyanidins are synthesized mainly before veraison, then decrease during ripening.

To calculate the mean degree of polymerization of proanthocyanidins (mDP), the sum of subunits contents in mg/kg (flavan-3-ols monomers and phloroglucynol adducts) was divided by the sum of all free flavan-3- oils monomers.

The mean degree of polymerization was calculated using response factors reported by Kennedy and Jones, (2001) of hydrolysis products of proanthocyanidins relative to the (+) - catechin, internal standard.

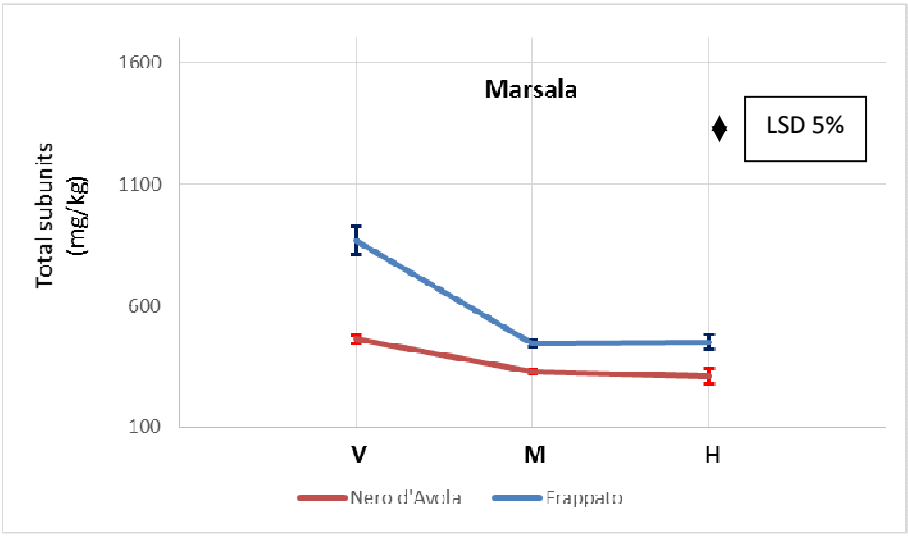
During ripening (tab. 12), in grapes skins of Sambuca site, the mean degree of polymerization of proanthocyanidins showed an higher increase than Marsala.

In Marsala, from first to last sampling, Nero d'Avola cultivar showed an increments of mDP, from 16,60 to 21,98; while, Frappato cultivar from 17,05 to 22,34.

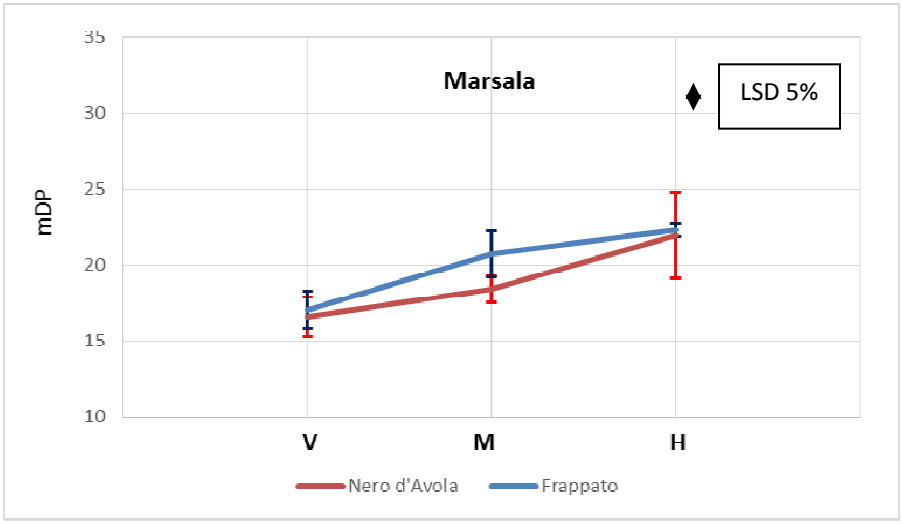
In Sambuca site, in Nero d'Avola cultivar, mDP goes from 22,33 to 26,59, instead in Frappato, from 26.38 to 31,58.

G x E combination		Sampling	Proanthocyanidins subunits						mDP	
Cultivar	Site		E* mg/kg	Std. Err.	T** mg/kg	Std. Err.	Tot. mg/kg	Std. Err.	value	Std. Err.
Nero d'Avola	Marsala									
		I	433,57	14,40	28,32	2,62	461,89	16,61	16,60	1,29
		II	309,78	8,66	20,14	1,61	329,92	10,05	18,43	0,87
		III	295,11	29,43	15,62	2,49	310,73	31,71	21,98	2,82
Frappato	Marsala									
		I	228,91	53,81	16,06	2,90	244,97	56,53	17,05	1,23
		II	263,31	13,22	14,20	1,82	277,51	15,04	20,75	1,53
		III	187,22	29,81	9,75	1,61	196,97	31,42	22,34	0,42
Nero d'Avola	Sambuca									
		I	526,89	111,10	25,22	2,94	552,11	113,77	22,33	2,74
		II	415,97	63,07	17,89	1,27	433,86	64,28	25,26	2,43
		III	334,05	45,59	14,18	0,22	348,23	45,45	26,59	3,72
Frappato	Sambuca									
		I	534,75	52,99	20,50	1,99	555,25	54,61	26,38	1,61
		II	378,79	16,61	17,38	1,37	396,17	15,54	26,50	3,11
		III	283,58	14,00	10,10	0,96	293,68	14,83	31,58	1,76

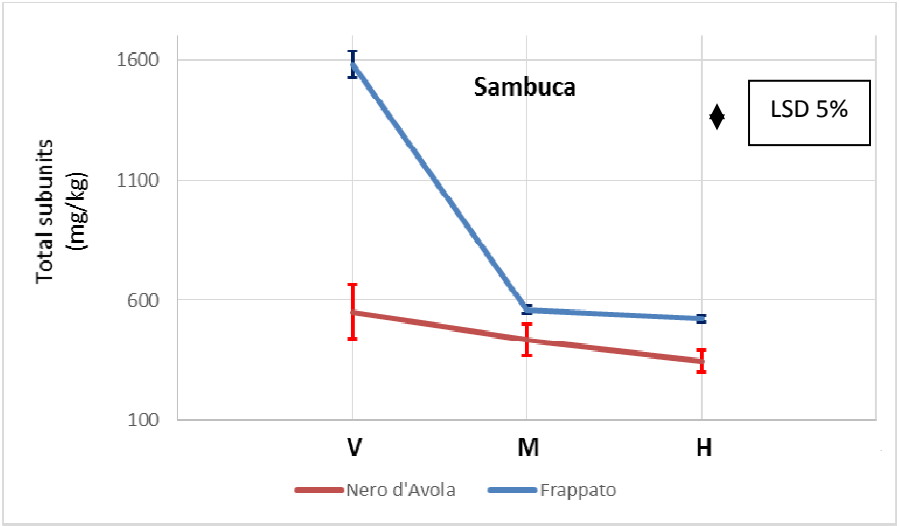
Table 12: Extention (E), terminal (T) and total (Tot) proanthocyanidins subunits and mean degree of polymerization (mDP) recorded in skins extract during ripening in each cultivar



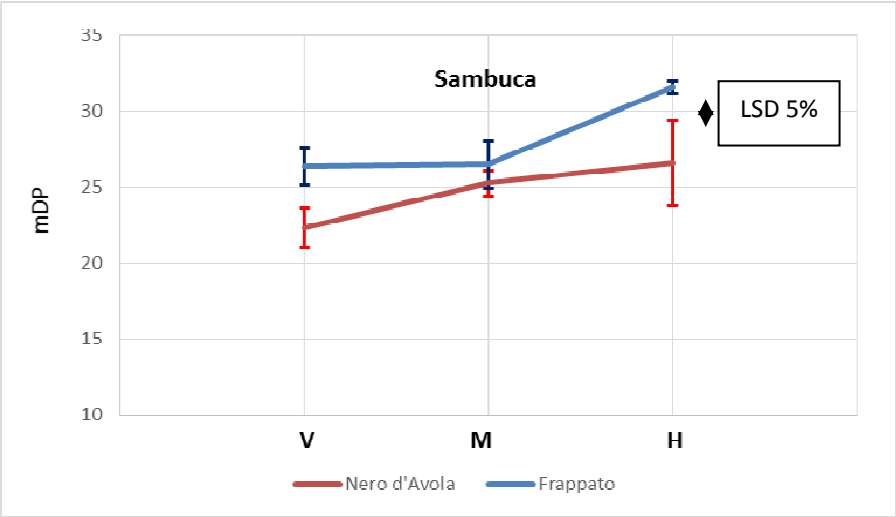
Graphic 17: evolution of total proanthocyanidins subunits in skins extract of Nero d'Avola and Frappato cultivar



Graphic 18: evolution of mean degree of polymerization in skins extract of Nero d'Avola and Frappato cultivar in Marsala site



Graphic 19: evolution of total proanthocyanidins subunits in skins extract of Nero d'Avola and Frappato cultivar in Sambuca site



Graphic 20: evolution of mean degree of polymerization in skins extract of Nero d'Avola and Frappato cultivar in Sambuca site

Biotype x site interaction - The total concentration of the subunits constituent and the mean degree of polymerization of the proanthocyanidins extracted from grape skins was higher in Sambuca biotypes than Marsala (tab. 13).

The apparent mean degree of polymerization of proanthocyanidins of this part of the berry was lower than the data reported in the literature; this could be attributed to the poor efficiency of the hydrolysis process and derivatization or not completely eluted with ethyl ether of the monomeric catechins from the C18 cartridge before recovery of oligomeric and polymer proanthocyanidins with methanol. Further investigations have to be carried out to clarify the discrepancies detected in this work.

For what concerns Nero d'Avola cultivar in Marsala site, A and B biotypes have been reported more subunits (respectively 362,73 and 368,50 mg/kg) compared to C (200, 95 mg/kg). Considering Frappato biotypes there weren't important differences, but B biotype was (237,10 mg/kg) basically superior to A (156,85 mg/kg).

In Sambuca site, subunits concentration of the B biotype of Nero d'Avola (503,22 mg/kg) prevailed on A biotypes (362,73 mg/kg) and C (200,95 mg/kg). There weren't difference between A and B biotypes of Frappato (respectively 331,76 and 275,60 mg/kg). The mean degree of polymerization of biotypes of Sambuca (tab. 13) was higher than Marsala.

In Marsala, for Nero d'Avola, the C biotype (mDP = 31,74) showed an degree of polymerization higher than A and B biotypes. Between A and B biotypes of Frappato (mDP respectively equal to 23,51 and 21,54) there weren't significant differences.

For what concerns Sambuca, B biotype of Nero d'Avola (mDP = 39,14) showed an highest degree of polymerization respect to A and C. As shown biotypes in Marsala, also in Sambuca, for Frappato biotypes, A and B (mDP amounting to 33,96 and 34,18) there weren't significant differences.

G x E combination			Proanthocyanidins subunits		mDP	
Cultivar	Biotype	Site	Tot. mg/kg	Std. Err.	value	Std. Err.
Nero d'Avola	*A*	Marsala	362,73	4,96	17,10	2,00
Nero d'Avola	*B*	Marsala	368,50	2,08	17,10	2,00
Nero d'Avola	*C*	Marsala	200,95	1,76	31,74	9,48
Frappato	*A*	Marsala	156,85	1,08	23,51	2,19
Frappato	*B*	Marsala	237,10	4,39	21,54	0,62
Nero d'Avola	*A*	Sambuca	246,69	1,85	17,73	2,10
Nero d'Avola	*B*	Sambuca	503,22	1,47	39,14	3,59
Nero d'Avola	*C*	Sambuca	294,79	2,53	22,90	3,26
Frappato	*A*	Sambuca	311,76	1,38	33,96	1,63
Frappato	*B*	Sambuca	275,60	2,97	34,18	10,01

Table 13: total (Tot) proanthocyanidins subunits and mean degree of polymerization (mDP) recorded in skins extract during ripening in each biotype

4.4 – EVALUATION OF MONOMERS CONSTITUENT, EXTENSION AND TERMINALS, THE MOLECULAR MASS AND HYDROPHOBIC INTERACTIONS OF WINE PROANTHOCYANIDINS (CHROMATOGRAPHIC DETERMINATIONS)

The data obtained in the laboratories of the Department of Viticulture and Enology, University of Fresno, California, have allowed it to gain important information about the characteristics of tannins in wines obtained whit grapes from two experimental fields. (tab. 14)

G x E combination			Phloroglucinolysis							GPC	Stickiness
Cultivar	Biotype	Site	EGC-P (mg/L)	C-P (mg/L)	EP-P (mg/L)	C (mg/L)	ECG-P (mg/L)	EC (mg/L)	subunits (mg/L	50% elution	(-kJ/mol)
Nero d'Avola	*A*	Marsala	51,86	23,77	384,85	86,79	21,55	37,75	606,56	14,06	4104,62
Nero d'Avola	*B*	Marsala	64,45	29,66	479,13	92,94	28,18	43,20	737,56	14,09	5536,29
Nero d'Avola	*C*	Marsala	41,91	22,76	312,57	81,54	16,79	32,49	508,07	14,38	2807,64
Frappato	*A*	Marsala	42,65	26,40	241,98	51,63	16,36	31,95	410,98	14,39	3609,94
Frappato	*B*	Marsala	49,85	32,13	289,36	65,01	12,51	41,74	490,60	14,24	4157,00
Nero d'Avola	*A*	Sambuca	68,14	22,25	413,86	95,09	22,21	49,38	670,93	14,36	3135,21
Nero d'Avola	*B*	Sambuca	95,93	25,07	445,53	126,59	28,85	44,51	766,47	14,46	3334,75
Nero d'Avola	*C*	Sambuca	41,01	16,31	287,67	71,77	21,92	25,83	464,51	14,68	3205,88
Frappato	*A*	Sambuca	105,06	26,76	350,04	63,34	24,68	27,16	597,05	14,47	4126,24
Frappato	*B*	Sambuca	153,11	71,56	712,41	172,86	44,28	97,91	1252,13	14,17	5731,67

Table 14: wine results recorded from cromatographic anlaysis, phloroglucynolysis, molecular mass (GPC) and stickiness (hydrophobic interactions)

Characterization of proanthocyanidins - The subunit composition of extension and termination monomers of wine proanthocyanidins confirmed, in qualitative terms, as reported by Kennedy and Taylor (2003). In fact, the chromatographic analysis to phloroglucinol revealed the presence of epicatechin, epigallocatechin, catechin and epicatechin-gallate 3O-gallate as extension subunits; catechin and epicatechin subunits as terminals units.

With the exception of wine obtained from grapes of C biotype of Nero d'Avola, there was a higher concentration of tannins in wines made of Sambuca than Marsala (tab. 15).

In all genotype x environment combinations, wines proanthocyanidins showed a strong dominance of epicatechin as extension subunitsunit (> 71% for Nero d'Avola and > 65% for Frappato). Over all, Frappato wines showed proanthocyanidins with a hgher percentage of epigallocatechin than Nero d'Avola wines. In fact, for Frappato wines, from Marsala grapes, biotypes A and B showed a percentage of epigallocatechin equal respectively to 12.05 and 11.98 % while, from Sambuca grapes, 19,700 and 14.54%.

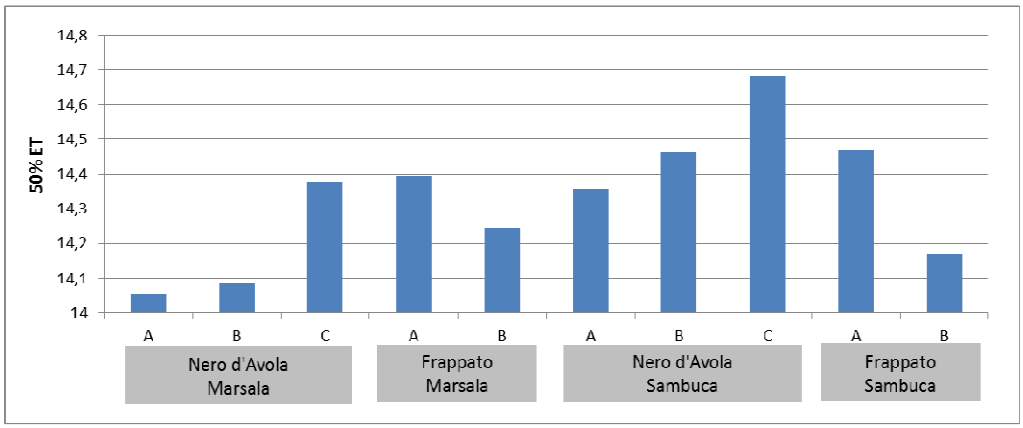
G x E combination			Extension units					Terminl units		
Cultivar	Biotype	Site	Concentration (mg/L)	EGC-P %	C-P %	EC-P%	ECG-P %	Concentration (mg/L)	C%	EC%
Nero d'Avola	*A*	Marsala	482,03	10,25	4,70	76,09	4,26	124,54	69,69	30,31
Nero d'Avola	*B*	Marsala	601,42	10,21	4,70	75,92	4,46	136,14	68,27	31,73
Nero d'Avola	*C*	Marsala	394,04	10,06	5,46	74,99	4,03	114,03	71,51	28,49
Frappato	*A*	Marsala	327,39	12,05	7,46	68,40	4,62	83,59	61,77	38,23
Frappato	*B*	Marsala	383,85	11,98	7,72	69,56	3,01	106,75	60,90	39,10
Nero d'Avola	*A*	Sambuca	526,46	12,42	4,06	75,42	4,05	144,47	65,82	34,18
Nero d'Avola	*B*	Sambuca	595,38	15,46	4,04	71,81	4,65	171,10	73,99	26,01
Nero d'Avola	*C*	Sambuca	366,91	10,70	4,26	75,07	5,72	97,60	73,54	26,46
Frappato	*A*	Sambuca	506,54	19,70	5,02	65,64	4,63	90,51	69,99	30,01
Frappato	*B*	Sambuca	981,35	14,54	6,80	67,66	4,21	270,78	63,84	36,16

Table 15: wines proanthocyanidins characterization

Molecular mass - The analysis based on gel permeation chromatography (GPC) were used for the determination of tannins molecular mass experimental wines.

Through this analysis, small molecules (small weight) permeate throughout the porosity of the chromatographic column with a high retention time. Big molecules are excluded from the porosity and are eluted with low retention times. In the graphic 21, low values of tannins retention time (50% elution) indicate high levels of molecular mass and opposite.

Basically, wines made from grapes of Marsala showed tannins with a higher molecular mass than Sambuca. Probably, during fermentation with skins and seeds (maceration), in musts of Sambuca, with highest sugar content than Marsala (more alcohol concentration), there was a greater extraction of tannins derived from seeds (low polymerized).



Graphic 21: 50% elution time (50 % ET) of proanthocyanidins

For wines of Marsala, the biotype A of Nero d'Avola (50% ET = 14,06) prevailed above the C biotype; instead, to Sambuca, biotype A (50% ET = 14, 36) prevailed on B and C biotypes. In Frappato wines in both experimental sites B biotype (Marsala: 50% ET = 14.24; Sambuca: 50% = 14.17 ET) showed tannins with a greater molecular mass than A.

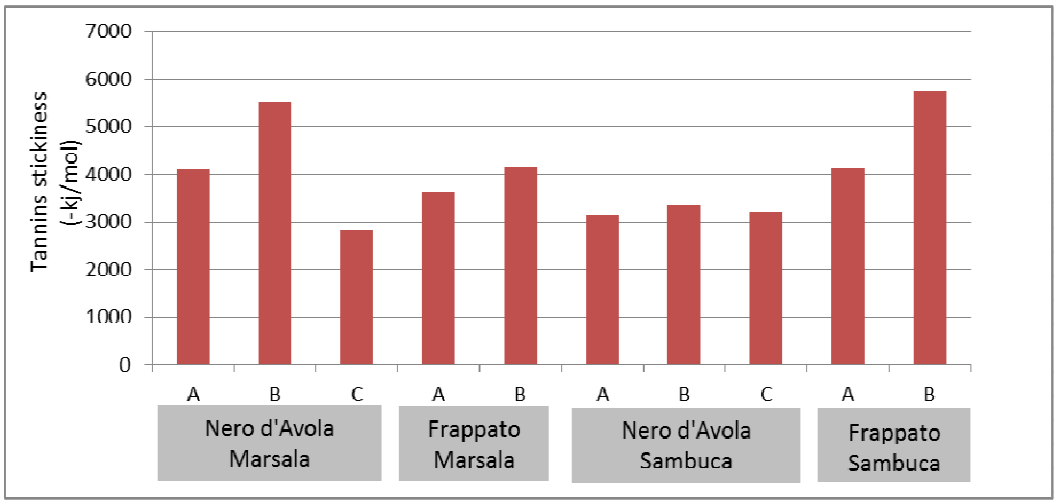
Hydrophobic interactions - The mouthfeel quality of red wine is an important management consideration, and tannins, which contribute astringency, are central to it. In addition to tannin, the matrix of red wine (e.g., ethanol, residual sugar, and polysaccharides) is known to have an impact on astringency perception. With regards to tannins and in addition to concentration, its composition (e.g., subunit composition, size distribution, pigment incorporation, and oxidation) is considered to affect tannin activity or its stickiness.

Stickiness is defined as the observed variation in the enthalpy of interaction between tannin and a hydrophobic surface. In this case, the hydrophobic surface is polystyrene divinylbenzene, and previous studies have found that stickiness variation with the tannin structure is similar to variations observed when using ITC to monitor the tannin interaction with poly-L-proline.

Stickiness measurements based on the thermodynamics of the tannin interaction with a hydrophobic surface suggest that this analytical approach has utility. The results show that, in wines made from Nero d'Avola, in Marsala site, tannins were able to create more hydrophobic interactions compared to wines of the same cultivar of Sambuca.

In Frappato, Sambuca wines prevailed over those of Marsala. Analyzing the results for biotype, at both experimental sites, the B biotype of Nero d'Avola and B biotype of Frappato prevailed over other biotypes of the same cultivar.

This appears in accordance with the tannins concentration in the respective wines. However, other factors, capable of influencing hydrophobic interactions amount, aren’t likely to be excluded.



Graphic 22: tannins stickiness

4.5 - WINES SENSORY ANALYSIS

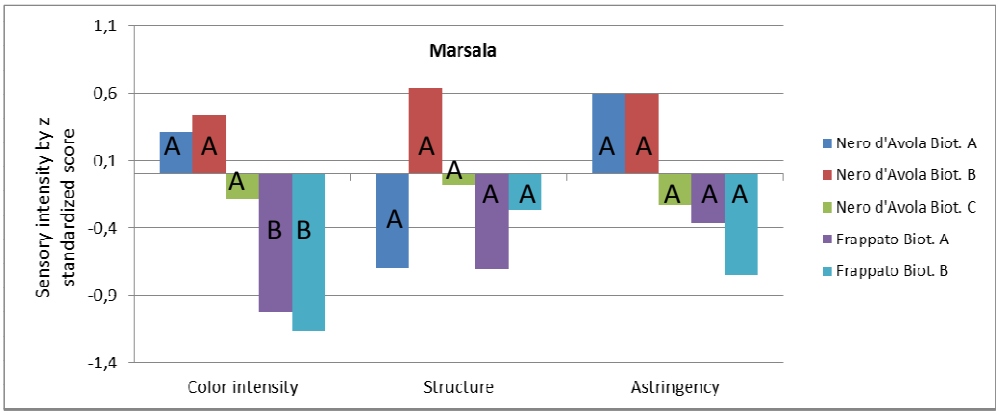
The sensory evaluation was carried out using a sensorial profile sheet based on a parametric non-structured assessment to detect color intensity, astringency and body characters. Four trained panelists evaluated the sensory notes of all samples. Data were then standardized for panelist by z transformation and statistically processed by ANOVA.

Based on the data obtained, visual and gustatory analysis shows that, for all the parameters considered, colour intensity, structure and astringency, values tend to be higher in the wines made from Sambuca than those of Marsala.

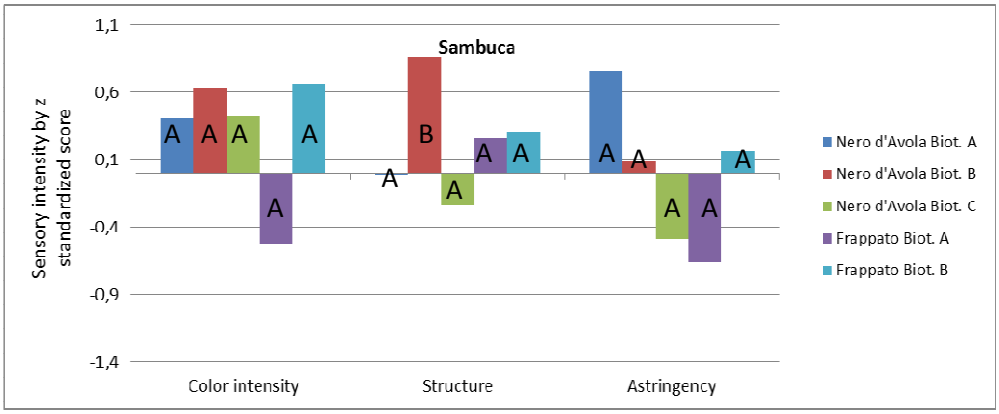
In particular, to astringency perception, within wines of Marsala there weren't important differences between biotypes; while, wines of Sambuca showed basically differences: A biotype of Nero d'Avola prevailed on B and C, instead, in Frappato cultivar, B biotype prevailed on A biotype.

G x E combination			Colour intensity	Structure	Astringency
Cultivar	Biotype	Site			
Nero d'Avo	*A*	Marsala	0,31	-0,70	0,59
Nero d'Avo	*B*	Marsala	0,44	0,64	0,60
Nero d'Avo	*C*	Marsala	-0,19	-0,08	-0,23
Frappato	*A*	Marsala	-1,03	-0,71	-0,37
Frappato	*B*	Marsala	-1,17	-0,26	-0,75
Nero d'Avo	*A*	Sambuca	0,41	-0,02	0,76
Nero d'Avo	*B*	Sambuca	0,63	-0,02	0,09
Nero d'Avo	*C*	Sambuca	0,42	-0,02	-0,49
Frappato	*A*	Sambuca	-0,52	-0,02	-0,66
Frappato	*B*	Sambuca	0,67	-0,02	0,17

Table 16: sensory analysis results



Graphic 23: sensory analysis of wines obtained from grapes of Marsala



Graphic 24: sensory analysis of wines obtained from grapes of Sambuca

5. CONCLUSIONS

The needs of growers and wineries are constantly changing, so it is important to know the characteristics of *Vitis vinifera* cultivars and selections in order to respond to market demands with wine production appreciated by consumers.

Results obtained in this work show, as indeed was already known from previous studies (Pigella et al., 1998) that the synthesis of flavanols in grape skins takes place under the control varieties. The proanthocyanidins evolution detected was different from flavanol monomers, as demonstrated by the progressive decrease of the two parameters and the increase of degree of polymerization deduced from the FRV/PI ratio during ripening.

Even chromatographic data obtained (vintage 2014) after acid hydrolysis in the presence of an excess of phloroglucynol (phloglucinolysis) confirm the decrease of proanthocyanidins during ripening and the increase of their degree of polymerization. The data confirm what has been observed by Anselmi (2012) on Nebbiolo and Barbera cultivar.

It is clear, in this work that environmental conditions play an important role on the accumulation and evolution of proanthocyanidins in grape skins. From data obtained in these two years of experience, it appears that the environmental conditions of Sambuca site, with higher average daily temperatures, determined, comparing during berry development and ripening the same cultivar and biotype, to a greater accumulation of proanthocyanidins, a greater decrease and a higher polymerization degree than the site of Marsala. These effects have been consequences on wines characteristics. Many scientific papers emphasize the role of temperature on the accumulation of secondary metabolites, such as those of Hawker (1982), Jones (1992), Ebadi et al. (1995) and Dokoozlian and Kliewer (1996) where relatively high temperatures (> 30°C) were able to increase the metabolic processes in the accumulation of polyphenols.

The phloroglucinolysis on skins proanthocyanidins was characterized by low conversion yields and it was not possible in this study to understand the reasons for the discrepancies between spectrophotometric data and chromatographic data.

The composition of proanthocyanidins extension units of Nero d'Avola and Frappato proved to be quite particular and confirmed, to qualitative aspect, experiences

of other authors (Cheynier et al. 1997a, Kennedy et al., 2001, Downey et al. 2003a). In the skins of these two varieties was detected in only the (+)-catechin as a terminal monomer. To extention subunit (-)-epicatechin was prevailed on other subunits, but in Frappato biotypes relevant percentage of (-)-epigallocatechin were recorded.

Despite some discrepancies between results obtained from the analysis performed on extracts of skins, it seems that in the site of Marsala, differences between biotypes of Nero d'Avola are less relevant than what we can see in Sambuca site, where the B biotype prevails on biotypes A and C both in terms of concentration of proanthocyanidins both to their degree of polymerization. While in Frappato cultivar there were not relevant differences between biotypes in both experimental site.

As wines, where tannins derived from skins and seeds are present, results indicate that, the biotype B of Nero d'Avola is characterized by a higher concentration of tannins and higher stickiness (tannins more reactive) than the others; this implies that wines from this biotype require an aging period, before consumption, in order to favourite the tannins evolution and decreasing of the astringency. Considering the degree of polymerization deduced from GPS analysis, the wines of A biotype of Nero d'Avola have been higher values than the other biotypes.

Taking into account Frappato, B biotype had more tannins and a higher degree of polymerization and stickiness than A in both experimental sites; therefore, it appears to be the biotype more suitable for production of wines demanding a period of aging; biotype A can be used for the production of wines ready to be drunk and easy to consume.

In conclusion, in this work, the first of this kind to be developed on Sicily native cultivars, we can say that the genotype x environment interactions have played a decisive role on the quality of grapes and wine. Data obtained have proven to be useful in understanding grapes physiological aspects, as a result of different environmental conditions, as well as to obtain practical knowledge to apply to wine-making level.

6. REFERENCES

Adams D.O. (2006). Phenolic and ripening in grape berries. Am. J. Enol. Vitic., 57, 3, 249

Adams, D.O., Harbertson, J.F. and Picciotto, E.A. (2004). Fractionation of red wine polymeric pigments by protein precipitation and bisulfite bleaching. In: Red wine colour: Revealing the mysteries. Eds. A.L. Waterhouse and J.A. Kennedy (American Chemical).

Alleweldt G. and Possingham J.V. (1988). Progress in grapevine breeding. Theor Appl Genet 75: 669-673.
Amrani-Joutei, K., Y. Glories, and M. Mercier. 1994. Localization of tannins in grape berry skins. Vitis 33:133-138.

Andrerson B. (2006). The wines of Italy. The Italian Trade Commission, New York.

Anselmi (2012). Tesi di laurea magistrale in Scienze Viticole ed Enologiche. Valutazione delle proantocianidine durante la maturazione dell'uva: contenuto e grado di polimerizzazione.

Ban, T., M. Ishimaru, S. Kobayashi, S. Shiozaki, N. Goto- Yamamoto, and S. Horiuchi. 2003. Abscisic acid and 2,4-dichlo- rophenoxyacetic acid affect the expression of anthocyanin biosyn- thetic pathway genes in 'Kyoho' grape berries. J. Hortic. Sci. Biotech. 78:586-589.

Bandielli R., Di Vecchi M., Ceseri L., Pierangoli L. and Borselli M., (2005). Obiettivi e risultati della ricerca condotta dall'Università di Firenze per la valorizzazione dei vitigni autoctoni. In: Convegno su Politiche di tutela e valorizzazione. Torino.

Bavaresco L., Fregoni C. (2001). Physiological role and molecular aspects of grapevine stilbenic compounds, in molecular biology & biotechnology of the grapevine. A.K. Roubelakis-Angelakis (Ed), Kluwer Academic Publishers.

Baxter, N.J.; Lilley, T.H.; Haslam, E.; Williamson, M.P. (1997). Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochemistry , 36, 5566-5577.

Bica D. (2007). Vitigni di Sicilia. Regione Siciliana. Assessorato Agricoltura e Foreste. Servizi allo Sviluppo. Palermo. Sicilia Agricoltura.

Boselli, E.; Boulton, R.B.; Thorngate, J.H.; Frega, N.G. (2004). Chemical and sensory characterization of doc red wines from Marche (Italy) related to vintage and grape cultivars. J. Agric. Food Chem., 52, 3843-3854.

Busse-Valverde, N.; Gómez-Plaza, E.; López-Roca, J.M.; Gil-Muñoz, R.; Fernández-Fernández, J.I.; Bautista-Ortiń, A.B. (2010). Effect of different enological practices on skin and seed proanthocyanidins in three varietal wines. J. Agric. Food Chem., 58, 11333-11339.

Cala, O.; Pinaud, N.; Simon, C.; Fouquet, E.; Laguerre, M.; dufourc, E.J.; Pianet, I. (2010). NMR and molecular modeling of wine tannins binding to saliva proteins: revisiting astringency from molecular and colloidal prospects. FASEB J., 24, 1-10.

Calò A., 2004 – Importanza dei vitigni locali per il settore vitivinicolo. – In: Recupero, conservazione e valorizzazione del germoplasma viticolo veneto. Veneto Agricoltura.

Carimi F., Mercati F., Abbate L., Sunseri F., (2010). Microsatellite analyses for evaluation of genetic diversity among Sicilian grapevine cultivar. Genet Resour Crop Evol 57: 703-719.

Carvalho, E.; Mateus, N.; Plet, B.; Pianet, I.; Dufourc, E.; de Freitas, V. (2006). Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins. J. Agric. Food Chem., 54, 8936-8944.

Castellarin S.D., Peterlunger E. (2007). Effetto dello stress idrico sulla biosintesi dei composti fenolici nella bacca di Vitis vinifera CV “MERLOT”. Quaderni di scienze viticole ed enologiche 2007, Università di Torino.

Castia T., Franco M.A., Mattivi F., Muggiolu G., Sferlazzo G., Versini G. (1992).

Castillo-Munoz N., Gomez-Alonso S., Garcia-Romero E., Hermosin-Gutierrez I. (2007). Flavonol profiles of Vitis vinifera red grapes and their single cultivar wines. J. Agric. Food Chem., 55, 992-1002.

Charlton, A.; Baxter, N.; Khan, M.; Moir, A.; Haslam, E.; Davies, A.; Williamson, M. (2002). Polyphenol/peptide binding and precipitation. J. Agric. Food Chem., 50, 1593-1601.

Chervin, C., A. El-Kereamy, J.P. Roustan, A. Latché, J. Lamon and M. Bouzayen. 2004. Ethylene seems required for the berry de- velopment and ripening in grape, a non-climacteric fruit. Plant Sci. 167:1301-1305.

Cheynier V., Rigaud J. (1986). HPLC Separation and characterization of flavonols in the skins of Vitis vinifera var. Cinsault. Am. J. Enol. Vitic., 37:4, 248-252.

Cheynier, V., C. Prieur, S. Guyot, J. Rigaud, and M. Moutounet. 1997b. The structures of tannins in grapes and wines and their in- teractions with proteins. In Wine: Nutritional and Therapeutic Benefits. T.R. Watkins (Ed.), pp. 81-93. ACS Symp. Ser. 661. American Chemical Society, Washington, DC.

Collesano G., (1998). Evoluzione varietale della viticoltura da vino in Sicilia. Vignevini 25, (7-8): 27-32.

Coombe B.G. (1987). Distribution of solutes within the developing grape berry in realation to its morphology. Am. J. Enol. Vitic., 38, 2, 120-127.

Cortell, J.M., M. Halbeib, A.V. Gallagher, T.L. Righetti, and J.A. Kennedy. 2005. Influence of vine vigor on grape (Vitis vinifera L. cv. Pinot noir) and wine proanthocyanidins. J. Agric. Food Chem. 53:5789-5797

Czochanska, Z., L.Y. Foo, and L.J. Porter. 1979. Compositional changes in lower molecular weight flavans during grape matura- tion. Phytochemistry 18:1819-1822.

Dallas, C.; Ricardo-da-Silva, J.M.; Laureano, O. (1996). Interactions of oligomeric procyanidins in model wine solutions containing malvidin-3- - glucoside and acetaldehyde. J. Sci. Food Agric., 70, 493-500.

Dan, D.H., and C.H. Lee. 2004. The effects of GA , CPPU and ABA applications on the quality of Kyoho (Vitis vinifera L. x Labrusca L.) Grape. Acta Hortic. (ISHS) 653:193-197.

Dangl G.S., Mendum M.L., Prins B.H., Walker M.A., Meredith C.P. and Simon C.J., (2001). Simple sequence repeat analysis of a clonally propagated species: a tool for managing a grape germplasm collection. Genome 44: 432-438.

de Freitas V.A.P., Glories Y., Monique A. (2000). Developmental changes of proanthocyanidins in grapes of red Vitis vinifera varieties and their composition in respective wines. Am. J. Enol. Vitic. 51, 397-403.

Demiglio, P.; Pickering, G.J. (2008). The influence of ethanol and pH on the taste and mouthfeel sensations elicited by red wine. J. Food Agric. Environ., 6, 143-150.

Di Stefano R., Borsa D., Ummarino I., Gentilini N., Follisi R. (2002). Evoluzione della composizione polifenolica di uve da cultivars diverse durante la maturazione. L’Enologo 38:10, 81-96.

Di Stefano R., Cravero M.C., Gentilini N. (1989). Metodi per lo studio dei polifenoli dei vini. L'Enotecnico 25, 83-89.

Di Stefano R., Mattivi F., Caburazzi M. Giustini E. Bonifazi L. (2008). Evoluzione della composizione fenolica dell’uva Sagrantino durante la maturazione. Riv. Vitic. Enol.

Di Stefano R., Moriondo G., Borsa D., Gentilini N., Foti S. (1994). Influenza di fattori climatici e colturali sul profilo antocianico varietale. L'Enotecnico 30:4, 73-77.

Dinnella, C.; Recchia, A.; Fia, G.; Bertuccioli, M.; Monteleone, E. (2009). Saliva characteristics and individual sensitivity to phenolic astringent stimuli. Chem. Senses, 34, 295-304.

Downey O.M., Harvey J.S., Robinson S.P. (2003) Analysis of tannins in seeds and skins of Shiraz grapes troughout berry development. Austr. J. Grape and Wine Res. 9, 15-27.

Downey, M.O., J.S. Harvey, and S.P. Robinson. 2004a. The effect of bunch shading on berry development and flavonoid accumula- tion in Shiraz grapes. Aust. J. Grape Wine Res. 10:55-73.

Downey, M.O., J.S. Harvey, and S.P. Robinson. 2004b. Flavonol accumulation and expression of a gene encoding flavonol synthase demonstrates light sensitivity of flavonol biosynthesis in grape- vines. In XXII International Conference on Polyphenols. A. Hoik- kala and O. Soidinsalo (Eds.), pp. 59-60. University of Helsinki.

Escribano-Bailo´n, M. T.; Guerra, M. T.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. (1995). Proanthocyanidins in skins from different grape varieties. Z. Lebensm. Unters. F. A., 200, 221-2.

Es-Safi, N.E.; Fulcrand, H.; Cheynier, V. (1999). Moutounet, M. Studies on the acetaldehyde-induced condensation of (-)-epicatechin and malvidin 3-O-glucoside in a model solution system. J. Agric. Food Chem**.**, 47, 2096-2102.

Falcetti, M. 1994. Le terroir. Qu'est-ce qu'un terroir? Pourquoi l'étudier? Pourquoi l'enseigner? Bull. OIV 67(2):246-275.

Fontoin, H.; Saucier, C.; Teissedre, P.-L.; Glories, Y. (2008). Effect of pH, ethanol and acidity on astringency and bitterness of grape seed tannin oligomers in model wine solution. Food Qual. Prefer., 19, 286-291.

Fournand D., Vicens A., Sidhoum L., Souquet J.M., Moutounet M., Cheynier V. (2006). Accumulation and extractability of grape skins tannins and anthocyanins at different advanced physiological stages. J. Agric. Food Chem., 54, 7331-7338.

Fulcrand, H.; Duenas, M.; Salas, E.; Cheynier, V. (2006). Phenolic reactions during winemaking and aging. Am. J. Enol. Viticult., 57, 289-297.

Galet P., (1991) – Precis d'ampelographie pratique. 6th edn. Charles Duhan Publishers.

Gawel, R. (1998). Red wine astringency: A review. Aust. J. Grape Wine Res. , 4, 74-95.

Gawel, R.; Francis, L.; Waters, E.J. (2007). Statistical correlations between the in-mouth textural characteristics and the chemical composition of Shiraz wines. J. Agric. Food Chem., 55, 2683-2687.

Gawel, R.; Iland, P.G.; Francis, I.L. (2001). Characterizing the astringency of red wine: A case study. Food Qual. Prefer., 12, 83-94.

Gawel, R.; Oberholster, A.; Francis, I.L. (2000). A 'mouth-feel wheel': terminology for communicating the mouth-feel characteristics of red wine. Aust. J. Grape Wine Res., 6,

Gladstones, J. 1992. Viticulture and Environment. 310 pp. Winetitles, Adelaide.

Hagerman, A.E. and Butler, L.G. (1978). Protein precipitation method for the quantitative determination of tannins. Journal of Agricultural and Food Chemistry 26, 809–812.

Hagerman, A.E.; Rice, M.E.; Ritchard, N.T. (1998). Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin16 (4f8) catechin (procyanidin). J. Agric. Food Chem., 46, 2590-2595.

Harbertson, J.F., Picciotto, E.A. and Adams, D.O. (2003). Measurements of polymeric pigments in grape berry extracts and wines using a protein precipitation assay combined with bisulphite bleaching. American Journal of Enology and Viticulture 54, 301–306.

Haslam, E. (1980). In Vino Veritas - Oligomeric procyanidins and the aging of red wines. Phytochemistry, 19, 2577-2582.

Haslam, E. (1998). Practical polyphenolics: From structure to molecular recognition and physiological action (Cambridge University Press: Cambridge).

Herderich, M.; Birse, M.; Dambergs, R.; Holt, H.; Iland, P.; Lattey, K.; Smith, P.A. (2006). Grape and wine tannins - an overview on current research, emerging applications, and future challenges. In Advances in Tannin and Tannin Management, Proceedings of AWRI

Herderich, M.J.; Smith, P.A. (2005). Analysis of grape and wine tannins: Methods, applications and challenges. Aust. J. Grape Wine Res., 11, 1-10.

Hufnagel, J.C.; Hofmann, T. (2008). Orosensory-directed identification of astringent mouthfeel and bittertasting compounds in red wine. J. Agric. Food Chem., 56, 1376-1386.

Huglin, P. 1978. Nouveau mode d'évaluation des possibilities héliothermiques d'un milieu viticole. C.R. Acad. Agric. Fr. 64:1117-1126.

Huglin, P., and C. Schneider. 1998. Biologie et écologie de la vigne. 370 pp. Lavoisier Tec & Doc, Paris.
IRVOS (2012). Istituto Regionale dei Vini e degli Oli. www.irvos.it

Jeong S. T., Goto-Yamamoto N., Hashizume K., Esaka M. (2006). Expression of the flavonoid 3'5'-hydroxylase genes and flavonoid composition in grape (Vitis vinifera). Plant Science,170, 61-69.
Jeong, S.T., N. Goto-Yamamoto, S. Kobayashi, and M. Esaka. 2004. Effects of plant hormones and shading on the accumulation of an- thocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. Plant Sci. 167:247-252.

Jordão A.M., Ricardo da Silva J.M., Laureano O. (2001). Evolution of catechins and oligomeric procyanidins during grape maturation of Castelão Francês and Touriga Francesa. Am. J. Enol. Vitic. 52:3, 230-234

Kallithraka, S.; Bakker, J.; Clifford, M.N. (1997a). Evaluation of bitterness and astringency of (+)-catechin and (-)-epicatechin in red wine and in model solution. J. Sens. Stud., 12, 25-37.

Kallithraka, S.; Bakker, J.; Clifford, M.N. (1997b). Red wine and model wine astringency as affected by malic and lactic acid. J. Food Sci., 62, 416-420.

Kallithraka, S.; Bakker, J.; Clifford, M.N. (1997c). Effect of pH on astringency in model solutions and wines. J. Agric. Food Chem., 45, 2211-2216.

Kallithraka, S.; Bakker, J.; Clifford, M.N.; Vallis, L. (2001). Correlations between saliva protein composition and some T-I parameters of astringency. Food Qual. Prefer., 12, 145-152.

Kennedy J. A., Matthews M. A., Waterhouse A. L. (2000a). Changes in grape seed polyphenols during fruit ripening." Phytochemistry 55: 77-85.

Kennedy J.A., Hayasaka Y., Vidal S., Waters E.J., Jones G.P. (2001). Composition of grape skin proanthocyanidins at different stages of berry development. J. Agric. Food Chem., 49, 5348-5355.

Kennedy J.A., Mathews M.A., Waterhouse A.L. (2002). Effect of maturity and vine water status on grape skin and wine flavonoids. Am. J. Enol. Vitic. 53, 268-274.
Kennedy J.A., Waterhouse A.L. (2000). Analysis of pigmented high-molecular-mass grape phenolics using ion-pair, normal-phase high-performance liquid chromatography. Journal of Chromatography A, 866, 25–34.

Kennedy, J. A.; Troup, G. J.; Pilbrow, J. R.; Hutton, D. R.; Hewitt, D.; Hunter, C. R.; Ristic, R.; Iland, P. G.; Jones, G. P. (2000b). Development of seed polyphenols in berries from Vitis vinifera L. cv. Shiraz. Aust. J. Grape Wine Res., 6, 244-254.

Kennedy, J.A. (2008). Grape and wine phenolics: Observations and recent findings. Cienc. Invest. Agrar., 35, 107-120.

Kennedy, J.A. and Jones, G.P. (2001). Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. Journal of Agricultural and Food Chemistry 49, 1740–1746.

Kennedy, J.A., M.A. Matthews, and A.L. Waterhouse. 2000. Changes in grape seed polyphenols during ripening. Phytochem- istry 55:77-85..

Kennedy, J.A., M.A. Matthews, and A.L. Waterhouse. 2002. The effect of maturity and vine water status on grape skin flavonoids. Am. J. Enol. Vitic. 53:268-274.

Kennedy, J.A.; Ferrier, J.; Harbertson, J.F.; Gachons, C.P.D. (2006). Analysis of tannins in red wine using multiple methods: Correlation with perceived astringency. Am. J. Enol.Viticult., 57, 481-485.

Kennedy, J.A.; Saucier, C.; Glories, Y. (2006). Grape and wine phenolics: History and perspective. Am. J. Enol. Viticult., 57, 239-248.

Kliewer, W.M. 1970. Effect of day temperature and light intensity on coloration of Vitis vinifera L. grapes. J. Am. Soc. Hortic. Sci. 95:693-697.

Landon, J.L.; Weller, K.; Harbertson, J.F.; Ross, C.F. (2008). Chemical and sensory evaluation of astringency in Washington state red wines. Am. J. Enol. Viticult., 59, 153-158.

Le Bourvellec, C.; Guyot, S.; Renard, C.M.G.C. (2004). Non-covalent interaction between procyanidins and apple cell wall material: Part I. Effect of some environmental parameters. BBA Gen. Subjects, 1672, 192-202.

Lee, C.B.; Lawless, H.T. (1991). Time-course of astringent sensations. Chem. Senses, 16, 225-238.

Levadoux L., (1956). Les populations sauvages et cultivèes de Vitis vinifera L. Ann Amelior Plan. 6: 59-118.

Li, H.; Guo, A.; Wang, H. (2008). Mechanisms of oxidative browning of wine. Food Chem., 108, 1-13.

Luck, G.; Liao, H.; Murray, N.J.; Grimmer, H.R.; Warminski, E.E.; Williamson, M.P.; Lilley,T.H.; Haslam, E. (1994). Polyphenols, astringency and proline-rich proteins. Phytochemistry, 37,357-371.

Macheix J.J., Fleuriet A., Jay-Allemand C. (2005). Les composés phenoliques des vègetaux. Presses polytecniques et universitaires romandes.

Mateus N., Silva A.M.S., Vercauteren J., de Freitas V. (2001). Occurrence of anthocyaninderived pigments in red wines. J. Agric. Food Chem., 49, 4836-4840.

Mateus, N.; de Freitas, V. (2001). Evolution and stability of anthocyanin-derived pigments during port wine aging. *J. Agric. Food Chem.*, *49*, 5217-5222.

Matthews, S., Mila, I., Scalbert, A., Pollet, B., Lapierre, C., Herve du Penhoat, C.L.M., Rolando, C. and Donnelly, D.M.X. (1997). Method for estimation of proanthocyanidins based of their acid depolymerization in the presence of nucleophiles. Journal of Agricultural and Food Chemistry 45, 1195–1201.

May, P. 1997. Using Grapevine Rootstocks. The Australian Perspective. 62 pp. Winetitles, Adelaide.

McRae, J.M.; Falconer, R.J.; Kennedy, J.A. (2010). Thermodynamics of grape and wine tannin interaction with polyproline: Implications for red wine astringency. J. Agric. Food Chem., 58, 12510-12518.

Menini U.G. (1998). Conservazione e utilizzazione sostenibile delle risorse citogenetiche per l'alimentazione e l'agricoltura: politiche e attività internazionali. Atti del 4° Convegno Nazionale, Alghero. Carlo Delfino editore.

Mercurio, M.D.; Smith, P.A. (2008). Tannin quantification in red grapes and wine: Comparison of polysaccharide and protein-based tannin precipitation techniques and their ability to model wine astringency. J. Agric. Food Chem., 56, 5528-5537.

Monagas, M.; Bartolome, B.; Gomez-Cordoves, C. (2005). Updated knowledge about the presence of phenolic compounds in wine. Crit. Rev. Food Sci. Nutr., 45, 85-118.

Montedoro, G. and Fantozzi, P. (1974). Dosage des tannins dans les moûts et les vins à l'aide de la methyl cellulose et evolution d'autres fractions phenoliques. Lebensmittel-Wissenschaft und Technologie **7**, 155–161.

Morata, A.; Gómez-Cordovés, M.C.; Calderón, F.; Suárez, J.A. (2006). Effects of pH, temperature and SO2 on the formation of pyranoanthocyanins during red wine fermentation with two species of Saccharomyces. Int. J. Food Microbiol., 106, 123-129.

Muganu M., Scossa A., Paolucci M., Bignami C., (2008). Conservazione del germoplasma viticolo e caratterizzazione di genotipi idonei all'impiego in viticoltura biologica.

Obreque-Slíer, E.; Peña-Neira, A.; López-Solís, R. (2010). Enhancement of both salivary proteinenological tannin interactions and astringency perception by ethanol. J. Agric. Food Chem., 58, 3729-3735.

Ojeda H., Andary C., Kraeva E., Carbonneau A., Deloire A. – 2002 – Influence of pre- and post-véraison water deficit on synthesis and concentration of skin phenolic compounds during berry growth of *Vitis vinifera* cv 'Shiraz'. *Am. J. Enol. Vitic.*, 53, 261-267.

Parish, M.; Wollan, D.; Paul, R. (2000). Micro-oxygenation – A Review. Aust. N. Z. Grapegrower Winemaker, 438a, 47-50.

Pastor del Rio J.L., Kennedy J.A. (2006). Development of proanthocyanidins in Vitis Vinifera L. cv. Pinot noir grapes and extraction into wine. Am. J. Enol. Vitic., 57:2, 125-132.

Pocock, K.F.; Sefton, M.A.; Williams, P.J. (1994). Taste thresholds of phenolic extracts of French and American oakwood: The influence of oak phenols on wine flavor. Am. J. Enol. Viticult., 45, 429-434.

Poncet-Legrand, C.; Cabane, B.; Bautista-Ortín, A.; Carrillo, S.; Fulcrand, H.; Pérez, J.; Vernhet, A. (2010). Tannin oxidation: Intra- versus intermolecular reactions. Biomacromolecules, 11, 2376-2386.

Ribéreau-Gayon P., Glories Y., Maujean and Dubourdieu D. (2003). Trattato di enologia II. Edagricole , Bologna.

Riou, C. 1994. Le déterminisme climatique de la maturation du raisin: application au zonage de la teneur en sucres dans la communauté européeenne. 319 pp. Office des Publications Officielles des Communautés Européennes, Luxembourg.

Roby, G., and M.A. Matthews. 2004. Relative proportions of seed, skin and flesh in ripe berries from Cabernet Sauvignon grapevines grown in a vineyard either well irrigated or under water deficit. Aust. J. Grape Wine Res. 10:74-82.

Roby, G., J.F. Harbertson, D.O. Adams, and M.A. Matthews. 2004. Berry size and vine water deficits as factors in winegrape com- position: Anthocyanins and tannins. Aust. J. Grape Wine Res. 10:100-107.

Russell, E.W. 1961. Soil Conditions and Plant Growth. Longmans, Green & Co., London.

Sáenz-Navajas, M.P.; Tao, Y.S.; Dizy, M.; Ferreira, V.; Fernández-Zurbano, P. (2010). Relationship between nonvolatile composition and sensory properties of premium Spanish red wines and their correlation to quality perception. J. Agric. Food Chem., 58, 12407-12416.

Saint-Cricq de Gaulejac, N., M. Augustin, N. Vivas, and Y. Glories. 1997. A biochemical approach to the evolution of procyanidins in grape seeds during the ripening of red grapes (Vitis vinifera L. cv. Merlot Noir). J. Wine Res. 8:159-167.

Salas, E.; Fulcrand, H.; Poncet-LeGrand, C.; Meudec, E.; Kohler, N.; Winterhalter, P.; Cheynier, V. (2005). Isolation of flavanol-anthocyanin adducts by countercurrent chromatography. J. Chromatogr. Sci., 43, 488-493.

Sarneckis, C.; Dambergs, R.G.; Jones, P.; Mercurio, M.; Herderich, M.J.; Smith, P. (2006). Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. Aust. J. Grape Wine Res., 12, 39-49.

Schubert A., Carra A., Riccomagno N. (2003). Biosintesi dei flavonoidi in Vitis vinifera. Quaderni della Scuola di Specializzazione in scienze viticole ed enologiche 2002-2003, Università di Torino.

Seguin, G. 1975. Alimentation en eau de la vigne et composition chimique des moûts dans les Grands Crus du Médoc. Phénomènes de régulation. Conn. Vigne Vin 9(1):23-34.

Seguin, G. 1986. “Terroirs” and pedology of vine growing. Experientia 42:861-873

Seguin, G. 1988. Ecosystems of the great red wines produced in the maritime climate of Bordeaux. In Proceedings of the Symposium on Maritime Climate Winegrowing. L. Fuller-Perrine (Ed.), pp. 36- 53. Department of Horticultural Sciences, Cornell University, Geneva, NY

Smith, A.K.; June, H.; Noble, A.C. (1996). Effects of viscosity on the bitterness and astringency of grape seed tannin. Food Qual. Prefer. , 7, 161-166.

Snoussi H., Harbi Ben Slimane M., Ruiz-Garcìa L., Martìnez-Zapater J.M., Arroyo-Garcìa R., (2004). Genetic relationship among cultivated and wild grapevine accessions from Tunisia. Genome 47:1211.1219.

Souquet J.M., Cheynier V., Brossaud F., Moutounet M. (1996). Polymeric proanthocyanidins from grape skins. Phytochemistry, 43:2, 509-512.

Spayed S.E., Tarara J.M., Mee D.L., Ferguson J.C. (2002). Separation of sunlight and temperature effects on the composition of Vitis vinifera cv. Merlot berries. Am. J. Enol. Vitic. 53, 171-182.

Squadrito M., Corona O., Ansaldi G., Di Stefano R. (2007). Relazioni fra i percorsi biosintetici degli HTCA, dei flavonoli e degli antociani nell'acino d'uva. Riv. Vitic. Enol., 60:3, 59-70.
Stoll, M. 2000. Effects of partial rootzone drying on grapevine physi- ology and fruit quality. Ph.D. thesis. Department of Horticulture, Viticulture and Oenology, University of Adelaide.

Tessier C., David J., Thip P., Boursiquot J.M., Charrier A., (1999). Optimizazion of the choice of molecular markers for varietal identification in Vitis vinifera L. Theor Appl Genet 98: 171-177.

Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. J. N. (1972). Plant proanthocyanidins. Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. J. Chem. Soc., Perkin I, 1387-1399.

Tosi E., Mirandola R. (2006). Recupero, conservazione e valorizzazione del germoplasma viticolo veronese. 29 novembre, 1 dicembre 2006; Villa Gualino - Torino.

Vidal, S.; Courcoux, P.; Francis, L.; Kwiatkowski, M.; Gawel, R.; Williams, P.; Waters, E. (2004d). Cheynier, V. Use of an experimental design approach for evaluation of key wine components on mouth-feel perception. Food Qual. Prefer., 15, 209-217.

Vidal, S.; Francis, L.; Guyot, S.; Marnet, N.; Kwiatkowski, M.; Gawel, R.; Cheynier, V. (2003). Waters, E.J. The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. J. Sci. Food Agr., 83, 564-573.

Vidal, S.; Francis, L.; Noble, A.; Kwiatkowski, M.; Cheynier, V.; Waters, E. (2004c). Taste and mouthfeel properties of different types of tannin-like polyphenolic compounds and anthocyanins in wine. Anal. Chim. Acta, 513, 57-65.

Vidal, S.; Francis, L.; Williams, P.; Kwiatkowski, M.; Gawel, R.; Cheynier, W.; Waters, E. (2004b). The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. Food Chem., 85, 519-525.

Vivar-Quintana, A.M.; Santos-Buelga, C.; Rivas-Gonzalo, J.C. (2002). Anthocyaninderived pigments and colour of red wines. Anal. Chim. Acta, 458, 147-155.

Waters, E.J.; Peng, Z.; Pocock, K.F.; Jones, G.P.; Clarke, P.; Williams, P.J. (1994). Solidstate 13C NMR investigation into insoluble deposits adhering to the inner glass surface of bottled red wine. J. Agric. Food Chem., 1994, 1761-1766.

Williams, V. M.; Porter, L. J.; Hemingway, R. W. (1983). Molecular weight profiles of proanthocyanidin polymers. Phytochemistry, 22, 569-572.

Winkler, A., J. Cook, W. Kliewer, and L. Lider. 1974. General Viticulture. 710 pp. University of California Press, Berkeley.

Yamane T., Jeong S.T., Goto-Yamamoto N., Koshita Y., Kobayashi S. (2006). Effects of temperature on anthocyanin biosynthesis in grape berry skins. Am. J. Enol. Vitic. 57, 54-59.

Zanchi, D.; Poulain, C.; Konarev, P.; Tribet, C.; Svergun, D.I.(2008). Colloidal stability of tannins: astringency, wine tasting and beyond. J. Phys. Condens. Matter, 20, 494224.

Zanchi, D.; Vernhet, A.; Poncet-Legrand, C.; Cartalade, D.; Tribet, C.; Schweins, R.; Cabane, B. (2007). Colloidal dispersions of tannins in water-ethanol solutions. Langmuir, 23, 9949-9959.

INDEX

ACKNOWLEDGMENTS

I owe my gratitude to Milan University that allowed me to live this great experience and to study abroad.
I cannot find words to express my gratitude to my supervisor Professor O. Failla for his precious advices, remarks, suggestions, enthusiasm and insight.
In addiction I thank the "Assessorato Regionale dell'Agricoltura, dello Sviluppo Rurale e della Pesca of Sicily region for providing all the infrastructures and all the equipments required for the research.
Finally, I dedicate this thesis to my parents and my sister, who have given me the opportunity of an education from the best institutions and support throughout my life.
It is with immense gratitude that I wish to thank my friend, Laura, who has helped and supported me to realization.