# Physiological responses of white grape berries to sunlight exposure

By

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# Declaration

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## Summary

Reflectance spectroscopy was used in 2013, to investigate about varietal behaviors to different agronomics condition: (i) composition and quantification of pigments by using non-invasive method; (ii) photosynthetic pigments assessment by new reflectance indices (iii) how different microclimatic bunch conditions could affect the appearance of sunburn. The experiment was performed in the Regional Research Station of Riccagioia (Lombardy region, Northern Italy), at the University of Milan, on 16 white grape accessions, during 3 phenological stages: pre-veraison (77 BBCH), veraison (81 BBCH) and harvest (89 BBCH). New specific indices for the evaluation and estimation of photosynthetic pigments were proposed on the basis of grape berry reflectance spectra. Validations with classical extraction analysis were done. About 200 berries were analyzed (over 1000 reflectance spectra were collected). First the chlorophyll *a* and *b* absorption maxima in the reflectance spectra were established: 675 and 650 nm respectively. These new equations are also able to discriminate between chlorophyll *a* and *b*. Indeed, the wavelengths of major interest for their absorption detection were identified. If chlorophyll quantifications were achieved directly from reflectance spectra, for carotenoids the absorption bands did not allow good reflectance correlations. Nonetheless, thanks to the physiological relation of photosynthetic pigments, the chlorophyll/carotenoid ratio was used to estimate carotenoid content. Because their proportion changes during berry development, the index coefficients can be adapted in relation to the BBCH phenological stage. These indices demonstrated good correlations with the destructive quantifications.

Also, the degradation intensity of the chlorophyll was different from that of the carotenoid during maturation, leading to a change in their absorption proportion throughout ripening. This finding allows suggesting that in white berries, the colour change during berry development is not related to the activation of a specific biosynthetic pathway, but is mostly the result of catabolic processes.

Chardonnay and Riesling showed different susceptibility to sunburn. The results suggest that for each variety, the timing of leaf removal during the day is fundamental to reducing

the appearance of brown color in the berry skin. In Chardonnay it would be better to avoid any leaf removal especially in pre-veraison, in the morning. Because Riesling was more susceptible during the afternoon, it would be recommendable to remove leaves, if necessary, during the early morning.

The aims of the last two years (2014-2015) data collection were: (i) to evaluate phenolics in Riesling and Chardonnay berries in response to sunlight exposure under different irrigation regimes; (ii) to study the relation of water stress to sunburn appearance. The experiment was carried out in the Columbia Crest vineyards (Columbia Valley, Washington State, USA). Chardonnay and Riesling berries were collected from two different vineyards. Two different irrigation regimes were applied in both vineyards. Full irrigation (FI): vines were irrigated to replace 100% crop evapotranspiration, from fruit set to harvest, with no water stress imposed. Deficit irrigation (DI): vines were irrigated to maintain a moderate water stress (stem water potential ($\Psi_{stem}$) at midday between -0.7 and -1 MPa) from fruit set to harvest. For compositional analysis two cluster exposures were considered: one exposed to direct sunlight (sun), and the other totally shaded from sunlight (shade). Skin flavonol, flavan3ol and proanthocyanidin content were analysed by HPLC methods. Total tannins were measured by spectrophotometer. Six temperature/light sensors per irrigation treatment were randomly installed on 3 sun and 3 shade bunches. In terms of absolute concentration in both varieties, several statistical tests indicate greater amount of variance accounted for by the effect exposure and phenological stages, and their interaction. No effect was due to the irrigation, in both varieties. Chardonnay had much higher flavanol concentration than Riesling. Chardonnay had much higher flavanols in the pre- veraison than during ripening. In Chardonnay the amount of monomers, dimers, trimers and polymers was greater in sun exposed berries than in shaded berries. Unlike in Chardonnay, in Riesling no flavan3ols monomers were detected by HPLC during the pre-veraison and veraison. In Riesling the amount of monomers, dimers and trimers was greater in sun exposed berries than in shaded berries. In both varieties flavonol concentrations were much higher in sun exposed berries than in

shaded berries, with absolute much higher concentration in Chardonnay. Flavonols increased during ripening, especially in the sun in both Chardonnay and Riesling. Under similar light conditions the difference in temperature within the two irrigation treatments in the sun, could be due to the less vigorous canopy of DI plants compared with FI plants, which overall lead to greater cluster exposure, in both varieties. Nonetheless, the temperatures were always lower in FI and higher in DI, supporting the hypothesis of a possible no (or little) effect on flavonol biosynthesis. Shaded-bunch temperatures were always lower as compared to sun exposed ones, as direct solar heating did not occur in the shaded-conditions.

# Dissertation

This dissertation is dedicated to all the future Ph. D student, to my family and to my friends.

*"Don't let the system get you down"*.

(Mattafix)

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# Chapter 1 Introduction: photosynthetic pigments and flavonoids

## 1.1 Berry structure and development

Grape berry is a non-climacteric fleshy fruit (Coombe and Hale 1973) composed of three different tissues, characterized by diverse biochemical profiles (Considine and Knox 1979, Hardie, *et al.* 1996). The exocarp or skin has an epidermis surrounded by a waxy cuticle and a hypodermis, formed by multiple layers of collenchymatous cells, containing most of the skin flavonoids (Adams, 2006). The mesocarp, also known as pulp, is composed of parenchyma cells, which are subjected to great expansion during fruit development. It contains phenolic hydroxycinnamates and a negligible amount of flavonoids (Douglas, 2006, Conde, *et al.* 2007). The endocarp is the innermost dermal tissue of the pericarp, surrounding the locules where the seeds develop. A great amount of grape flavanols localize in some layers of the seed coat, as well as in the exocarp (Adams, 2006). Berry growth is a process that follows a double sigmoid curve (Coombe, 1992), involving three different phases (Conde, *et al.* 2007). In the first phase there is the berry formation, characterized by pericarp cell division, from which the final size of the berry will depend. Also, it is correlated with the growth rates of seed development. During this stage the berry is green and hard and the compound that is principally accumulated is malic acid (Coombe and McCarthy 2000), then to be decreased in the second part of the growing season (Sweetman, *et al.* 2012). Moreover, hydroxycinnamates, tannins and other phenolic precursors are biosynthesized. Through the lag phase the cell expansion slows and in the seeds mature (Conde, *et al*. 2007). During this first growing cycle, the vascular flow into the berry derives from both xylem and phloem (Coombe and McCarthy 2000). The end of the lag phase is characterized by veraison. It was demonstrated that the changes occurring from this stage onwards, are promoted by the accumulation of the hormone abscisic acid (Wheeler, *et al.* 2009). Ripening is characterized by cell expansion and softening of the berry, sugar accumulation, pigmentation and decreasing of the organic acid levels (Zoccatelli, *et al.* 2013). During this second cycle of growth the vascular flow into the berry is mostly attributed to phloem sap (Coombe and McCarthy

2000). Also during this phenological stage there are accumulations in aroma precursors and compounds (Lund and Bohlmann 2006).

## 1.2 Composition in white *Vitis vinifera* grapes

### *1.2.1 Photosynthesis in leaves and fruits*

Higher plants, algae, and phototropic bacteria convert solar energy into high-energy molecular species reducing power (Blankenship, 2002). 10-100 picoseconds is the timescale that occurs from solar energy capturing by the light harvesting complexes and their funnel to the reaction centers (Van Grondelle, *et al.* 1994). The crucial stage of the photochemistry takes place in the thylakoid membrane bound pigment-protein complexes, the photosystem II and I reaction centers (PSII RC, PSI RC). These photosystems are connected through an electron transport chain, which include the cytochrome $b_6f$ membrane complex. The electron transfer is coupled to proton transportation from the stroma into the lumen aqueous phase. The PSII RC and PSI RC use the energy of an absorbed photon to conduct the electron transfer reactions. In particular in the PSII RC the electron transfer is associated to the generation of a protonic gradient from the splitting of $H_2O$ and the reduction of the plastoquinone. This produced gradient finally drives the formation of adenosine triphosphate (ATP). In the PSI RC the transmembrane electron transfers occur from the plastocyanin (in the thylakoid lumen) to the ferredoxin (on the stromal side of the membrane); it is linked to the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to create NADPH. The NADPH and ATP so formed are then used to fix and reduce the carbon dioxide, in order to produce sugars (Anna, *et al.* 2013). The overall reaction of photosynthesis is:

$$6CO_2 + 12H_2O \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O$$

$\Delta G = + 2840$ kJ $mol^{-1}$ (1mol glucose = 180 g)

The PSII RC contains six chlorophylls, two pheophytins, two $\beta$-carotenes and one cytochrome b-559 (Romero, *et al*. 2011, Gounaris, *et al.* 1990). The reaction centers are surrounded by about 200 chromophores (absorbing solar photons) bound in light

harvesting complexes (Scholes, *et al.* 2011). The energy captured is stored through the electronic excitation states of the same chromophores and then transferred within and among the light harvesting proteins to finally reach a reaction center (Anna, *et al.* 2013, Van Grondelle, *et al.* 1994). In higher plants the chromophores in the light harvesting complexes include chlorophylls and carotenoids, such as *β*-carotene and lutein. In particular, the major light harvesting complex (LHCII, associated with PSII RC) alone is able to capture 20% of the incident light at 625 nm (Scholes, *et al.* 2011). The LHCII contains 42 chlorophylls (24 chlorophyll *a* and 18 *b*) (Romero, *et al.* 2011). Even the PSI RC has light harvesting complexes associated with it (LHCI), which have a similar structure to the LHCII (Dreyfuss and Thornber 1994). LHCI bind chlorophyll *a* and *b*, and xanthophylls. More than 100 chlorophyll molecules were estimated in the LHCI for each PSI RC (Boekema, *et al.* 1990). The energy of the singlet excited state of most of the antenna chlorophylls that serve the PSII RC is close to that of P680, that of the one that serves the PSI is close to P700 (Van Grondelle, *et al.* 1994). There is a physical separation in the thylakoid membrane between the two reaction centers and their antenna complexes. This is mostly in order to prevent the PSI draining off the excitation energy from PSII, but also to regulate and to balance the energy distribution between them at a particular light intensity and spectral composition (Anderson and Anderson 1988). In case of an imbalance in favor of the PSII RC, there is more reduction of plastoquinone, which leads to the activation of the LHCII kinase. Thus the phospho-LCHII dissociates from PSII to the PSI to balance the energetic distribution. In case of over excitation of the PSI, the plastoquinone gets oxidized and the kinase inactivated (Allen, 1992). Instead, if the light absorption exceeds the capacity of the photochemistry, the antenna chlorophylls triplet can be formed and this leads to sensitizing the production of singlet oxygen (Owens, 1994). The PSII RC and other components of the photosynthetic membrane can be damaged by the singlet oxygen, leading to photoinhibition or decrease of photosynthetic efficiency (Ledford and Niyogi 2005). To reduce or minimize as much as possible these destructive mechanisms, a plant may change the orientation of leaves or chloroplasts (Kasahara, *et al.* 2002) or could start shrinking the antenna size of PSII RC

(Escoubas, *et al.* 1995). Even a redistribution of the excess absorbed energy to alternative electron transport pathways can occur, either to water via the water- water cycle (Asada, 2000) or to photorespiratory pathway (Wingler, *et al.* 2000) or to oxygen via a chlororespiratory pathway (Aluru and Rodermel 2004). Plus, the non-photochemical quenching (NPQ) mechanism is quickly activated to dissipate energy as heat (Demming-Adams and Adams 1992, Holt, *et al.* 2004). A decrease in net photosynthesis and stomatal conductance was reported for grapevine leaves and other species, under condition of high irradiance and vapor pressure deficit (Gomez del Campo, *et al.* 2004, Moutinho-Pereira, *et al.* 2004), in concomitance to a midday to afternoon depression in the leaf water potential (Chaves, *et al.* 2007). Nonetheless, the decrease in net photosynthesis was also associated with other factors, such as the accumulation of abscisic acid in the petiole xylem, an increase in the pH of xylem (Rodrigues, *et al.* 2008) and substomatal carbon dioxide concentration (Quereix, *et al.* 2001). A decrease in the quantum efficiency of the PSII was also demonstrated by using remote sensing of chlorophyll fluorescence (Flexas, *et al.* 2000). Different soil water stress or irrigation regimes could also downregulate the net photosynthesis (Flexas, *et al.* 2002, Medrano, *et al*. 2002).

The above cited bibliography mostly concerns studies on leaves, as they are considered the main sources of photosynthate production (Aschan and Pfanz 2003). In grapevines, the berry is known to be a sink organ. Indeed, its development is strictly dependent on the photosynthates imported from the productive leaves (Blanke and Lenz 1989). However, the fruit itself is able to contribute slightly to the overall carbon gain, as in the grapevine berry (Hale, 1962) and as in reproductive organs of other species (Blanke and Lenz 1989, Aschan and Pfanz 2003). However, the chloroplast density is much lower in fruit tissues as compared to leaves, thus the photosynthetic rates per unit area is reduced (Aschan and Pfanz 2003). The outer epidermal layers of the fruit contain stomata up to 100 times lower density than that of the leaves (Moreshet and Green 1980). Moreover this number remains constant, but the density decreases during ripening, because of fruit surface expansion. Plus, the stomata become non-functional (Rogiers, *et al*. 2004). Indeed, the amount of stomata are set early at anthesis (Blanke and Lenz 1989). In early fruit development the

stomata regulates the rate of carbon dioxide and water exchange. In ripening fruit the cuticle and the lenticels control the diffusive resistance to carbon dioxide (Schreiber and Riederer 1996). It was shown that the morphology of grape berry stomata, surrounded by a conspicuous tissue, reduces transpiration losses and maintains fruit turgor (Blanke, *et al*. 1999).

If the leaves are characterized as having tissues able to do photosynthetic assimilation mostly using carbon dioxide from the atmosphere, the berries recycle the internal carbon dioxide produced by respiratory mechanisms (Aschan and Pfanz 2003). Because of the surface to volume ratios of the berry, this fixation mechanism of internally respired carbon could be an adaptation to improve the carbon balance (Blanke and Lenz 1989). The morphology of fleshy fruits imposes physical constrictions to light penetration into the inner tissues (Breia, *et al.* 2013). Only 2% of the photon flux density was found to reach the internal regions of the fruits (Aschan and Pfanz 2003). Moreover, not all the light filtered by green skins is effectively absorbed by chlorophylls. By studying the effect of saturation pulse light intensity on chlorophyll fluorescence values, it was demonstrated that chlorophylls are much less abundant in the mesocarp than in the exocarp, or in the seed integument, which actually showed the highest chlorophyll content (Breia, *et al.* 2013). Indeed, in grape berry (Breia, *et al.* 2013), as in other fruits (Ruuska, *et al.* 2004), it was demonstrated that the capacity of cell layers surrounding the seeds to photosynthesize, could also contribute with Calvin cycle-derived precursors for the synthesis of secondary metabolites as, for example, tannins. Naturally, grape clusters are moderately shaded by leaves, showing mean light intensity of 30 to 50 µmol photons $m^{-2}$ $s^{-1}$ and about 1 to 5 µmol photons $m^{-2}$ $s^{-1}$ for those heavily shadowed in the canopy (Bergqvist, *et al.* 2001). Using saturation pulse light intensity, it was suggested that 2500 µmol photons $m^{-2}$ $s^{-1}$ were able to close the PSII RC, while about 5000 µmol photons $m^{-2}$ $s^{-1}$ determined significant photoinibition in the exocarp, as well as in the seed integument (Breia, *et al.* 2013). The same authors demonstrated that the exocarp of grape berry, as compared to the mesocarp, showed the highest photochemical competence, having the higher photosynthetic efficiency and capacity and the lowest susceptibility to

photoinhibition. The seed outer integument was characterized by photochemical efficiency similar to that of the outermost layer of skin tissues, even if it was revealed to be more susceptible to photoinhibition than the exocarp.

### *1.2.2 Photosynthetic pigments*

The photosynthetic pigments are compounds able to absorb light in the visible part of the electromagnetic spectrum. Their color is related to the chromophore structure, in which the catching of the solar energy determines the excitation of an electron from its ground state into an excited state. Not all the energy is usually absorbed: a part could be reflected and/or transmitted. In general, the structure of the chromophores defines the overall pigment classification. The carotenoids belong to the class of chromophore with conjugated systems; while the chlorophylls to the metal-coordinated porphyrins (Lichtenthaler, 1987). Nevertheless, because of their common isopentenoid pathway, the carotenoid and the chlorophyll, also referred to as prenyl pigments, belong to the group of isoprenoid plant lipids.

Chlorophylls are mainly present in the chloroplasts of higher plants and most algae. Chlorophyll *a* as major pigment, achieves two main functions: light harvesting and energy transfer from the distant antenna to the reaction center (Bricker, 1990). Chlorophyll *b* is an accessory pigment. The chlorophylls occur in a ratio (*a*/*b*) 3:1. Sun exposed plants have high-light chloroplasts, and exhibit ratio of 3.2 to 4. Shade plants (low-light chloroplasts) possess lower values for *a*/*b* ratio 2.5 to 2.9 (Lichtenthaler, 1987). Chlorophylls have a porphyrin ring (a characteristic cyclic arrays structure with tetrapyrrole) bonded to a magnesium atom. Chlorophyll *a* differs from *b* in having a methyl (-$CH_3$) group on ring II instead of a formyl one (-CHO) (fig. 1). The phytyl chain gives chlorophylls (*a*, *b*) a hydrophobic character (Dey and Harborne 1997).

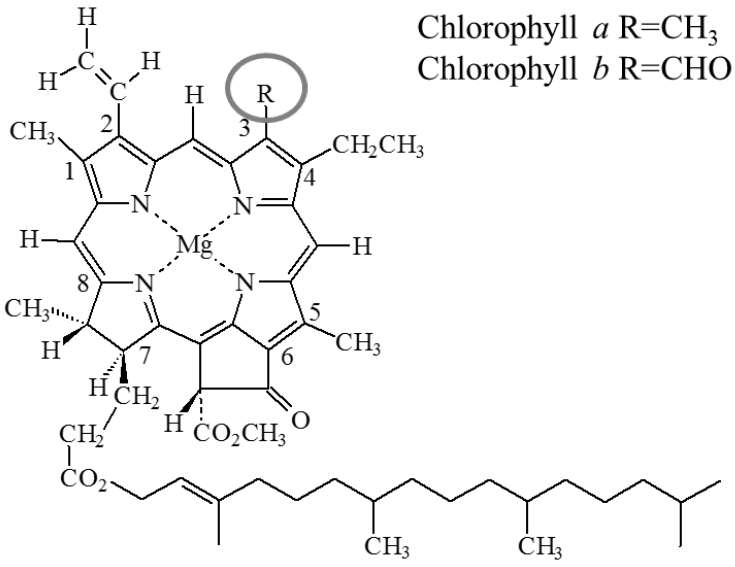


*Figure 1 Structure of chlorophyll a and b.*

The chlorophyll, when excited by visible light, arises a first ($S_1$) and a second ($S_2$) vibrational electronic singlet state, which leads it to absorb light respectively in the red (peak at 670 to 680 nm) and blue region (435 to 455 nm respectively) of the visible spectrum. This results in a characteristic green color when the chlorophylls are in solutions. The relaxation to a lower vibrational state of both $S_1$ and $S_2$, may occur by thermal emission, fluorescence or intersystem crossing to the triplet state of chlorophyll $^3Chl^*$. When it is in the $S_1$ excitation state, the chlorophyll *a* is more reductant than in the ground state. This activates the electron transfer process to an acceptor within the two photosystems. If the excitation energy level $S_1$ of the chlorophyll *a* is not transferred to another molecule, a triplet state of chlorophyll $^3Chl^*$ is generated in few nanoseconds. This triplet can react with $O_2$ to produce the highly reactive species $^1O_2^*$. Within the functions of the xanthophyll present in the light harvest complexes there are not only light absorption, but also the quenching of this triplet of chlorophyll.

From an eco-physiological point of view, the chlorophyll as photosynthetic light harvesting and energy transduction in higher plants, and the carotenoid, are considered to give indirect information for estimating the plant nutrient status (Filella *et al.* 1995;

Moran *et al.* 2000), the water stress and the tissue senescence (Hendry *et al.* 1987, Merzlyak *et al.* 1995). On these bases they could be used for an indirect estimation of berry radiative condition during ripening.

Carotenoids belong to the group of red or yellow pigments which absorb light between 450-570 nm in the visible light range (Baumes, *et al.* 2002). In natural sources, carotenoids occur mainly in the all-trans (all-E) configuration (Razungles, *et al.* 1987). Isomerization of trans-carotenoids to cis-isomers (all-Z) is promoted by contact with acids, heat treatment, and exposure to light (Baumes, *et al.* 2002, Razungles, *et al.* 1988). Lutein and $\beta$ carotene represent 85% of all the carotenoids content in mature grapes. Other minor xanthophylls present are: neoxanthin, violaxanthin, lutein 5-6-epoxide, zeaxanthin, necrome, flavoxanthin, and luteoxanthin.

In photosynthetic tissues of higher plants the carotenoid performs two crucial functions: photo-protection and light-harvesting (Palett and Young 1993). For example, they absorb the blue green light not absorbed by the chlorophylls; stabilize the pigment-protein structure in the thylakoid membrane, and are involved in the energy transfer to and from the chlorophylls (Dey and Harborne 1997). From an enological point of view, carotenoid breakdown products, the norisoprenoids (Baumes, *et al.* 2002), play important roles in wine aroma, because of their low olfactory perception threshold (Mendez-Pinto 2009). Descriptors such as rock-rose, violet, quince, camphorwood and kerosene are related to these compounds. Of course, many other factors contribute to the norisoprenoid bouquet in wines (e.g. fermentation process and wine storage conditions), but the grape carotenoid concentration and profile are considered to be determinant factors (Mendez-Pinto, 2009). Furthermore, both carotenoid and chlorophyll are also potential indicators of berry ripeness (Baumes, *et al.* 2002). The coloration of the fruits during ripening is considered to have an attractive function for dispensers (Willson and Whelan 1990). During the early stage of development grape berries are green, then upon ripening their colors change to yellow, pink or red, depending on the variety. The ability to photosynthesize in ripening fruit is reduced due to either decrease and/or absence of photosynthetic pigments, or

masking by other colored pigments (Blanke and Lenz 1989). In terms of chlorophyll concentrations, in Merlot grape the chlorophyll *a* (expressed as a sum with pheophytin *a*) was found to be the most abundant pigment throughout ripening, with just a 50% degradation observed at harvest (on a per berry fresh weight base). While the chlorophyll *b* (expressed as sum with pheophytin *b*, and pyropheophytin *b*) showed a degradation of 20 to 30% from its original concentration (5.2 - 6.9 μg/berry, to 3.7 - 4.5 μg/berry at harvest) (Kamffer, *et al.* 2010). Within the xanthophyll group the zeaxanthin was demonstrated to occur at very small concentrations during pre-veraison (0.15-0.3 μg/berry fresh weight) and to finally decrease to negligible amounts at harvest (Kamffer, *et al.* 2010). In the literature there is a discrepancy about the lutein content of berries during maturation. Most authors suggested a decrease in its level from veraison to harvest (Oliveira, *et al.* 2004, Razungles, *et al.* 1996). Others found an increase during the first part of ripening (pre-veraison to post-veraison) (Kamffer, *et al.* 2010). Little data exists for pre-veraison grapes. Instead there is agreement about the *β*-carotene compound, which showed an increase from pre-veraison to veraison, to then decline (Kamffer, *et al.* 2010, Oliveira, *et al.* 2004, Razungles, *et al.* 1996). In general, while most of the photosynthetic pigments content decreased during berry development, the 5, 8-epoxy-*β*-carotene, an oxidation product of *β*-carotene, increased as ripening progressed (Kamffer, *et al.* 2010). Overall warm temperatures and high exposure to sunlight increase carotenogenesis, but may also promote carotenoid photo-degradation (Rodriguez-Amaya, *et al*. 2008).

### *1.2.3 The Flavonoids*

More than 6000 different flavonoids were identified within natural products (Ferrer, *et al.* 2008). In grape, flavonoids are the most abundant phenolic compounds. All flavonoids appear as $C_{15}$ skeleton in their aglycone form (fig. 2a).

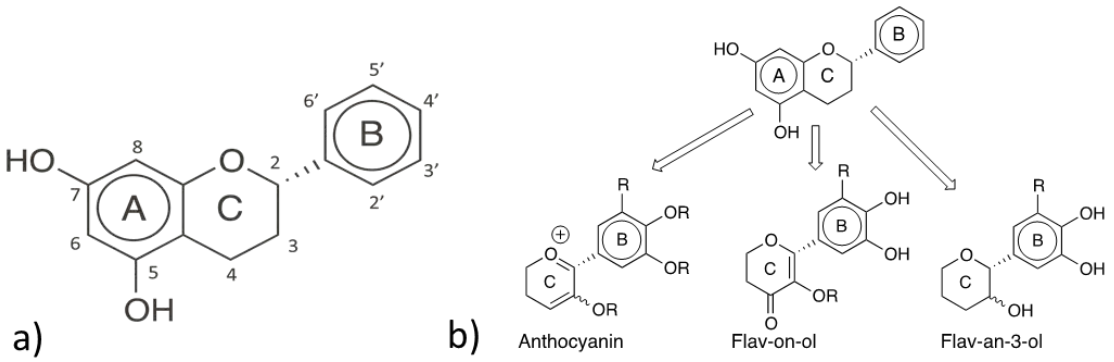


*Figure 2 a) Basic flavonoid skeleton with labelling convention. Modified from (Kennedy, et al. 2006); b) Most relevant classes of flavonoids, in the figure are shown the C and B ring (courtesy J. Habertson).*

In general the flavonoids are glycosylated. Also, some of them bear acylated aliphatic and aromatic acids. They were classified according to the degree of oxidation and substitution of the third central pyran ring (*C* ring). The most relevant classes are: anthocyanins, flavonols, flavan-3-ols and proanthocyanidins or condensed tannins (Schwinn and Davies 2004) (fig. 2 b). In addition to these, other major subgroups were also proposed: chalcones, flavones and flavandiols (Winkel-Shirley, 2001). For all of them the precursor is a flavanone, the naringenin. It yields from the polyketide condensation reaction of p-4-cumaryl-CoA with three acetate units from malonyl-CoA. The next step in the pathway is the cyclization of naringenin chalcone under the chalcone isomerase (CHI) to produce the flavanone naringenin. The hydroxylation of the C3 position leads to the formation of dihydroflavonol. The *B* ring hydroxylation pattern of the dihydroflavonol depends mostly from the presence and/or absence of the enzymes F3'H: Flavanone 3'Hydroxylase and F3'5'H: Flavanone 3'5'Hydroxylase, leading to the procyanidins and prodelphynidins as flavonoids. From the dihydroflavonol, the biosynthesis branches to the specific pathway of flavonol under the flavonol synthase (FLS), or yields leucocyanidin by the dihydroflavonol-4-reductase (Dey and Harborne 1997). Leucocyanidin leads to 2, 3-trans flavan-3-ol, and cyanidin leads to 2, 3 cis flavan-3-ol and anthocyanin (in red grape) (Mattivi, *et al.* 2006) (fig. 3).

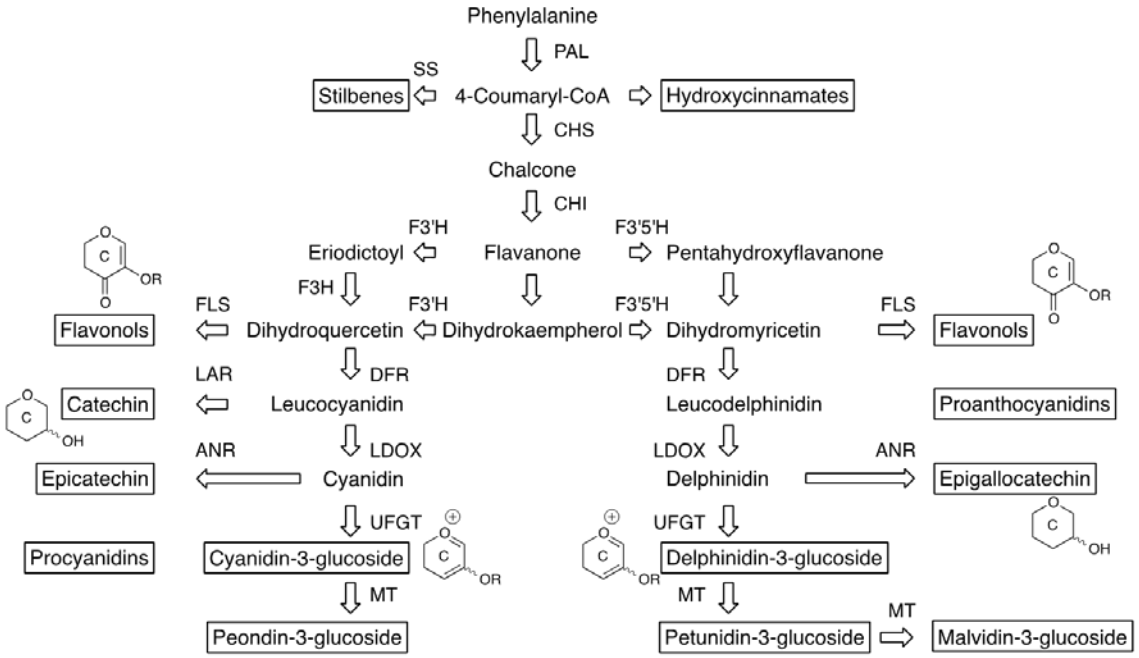


*Figure 3 Flavonoid pathway. SS: Stilbene synthase, PAL: Phenylalanine ammonia lyase, CHS: Chalcone synthase, CHI: Chalcone isomerase, F3'H: Flavanone 3'hydroxylase, F3'5'H: Flavanone 3'5'hydroxylase, F3H Flavanone 3 hydroxylase, DFR: Dihydroflavonol reductase, FLS: Flavonol sythase, LDOX: Leucoanthocyanidin dioxygenase, UFGT: UDP-glucose:flavonoid 3-O-glucosyltransferase, LAR: Leucocyanidin reductase, ANR: Anthocyanidin reductase, MT: Methyltransferase (courtesy J. Habertson).*

Usually, flavonoids are true end products. However, oxidation, demethylation and hydration can occur. For example, upon pathogen attack or senescence of tissues, dark-brown coloured substances may appear as a consequence of flavonoid oxidation (Dey and Harborne 1997, Pourcel, *et al.* 2006). This latter process could lead either to polymerization or to destruction to form hydroxybenzoate as initial catabolite, under peroxidase catalysis.

Two classes of genes are involved in the flavonoid biosynthesis in grapevine: the structural class and the regulatory class. The first group encodes for the enzymes required for the synthesis of each compound, whereas the second group regulates the spatial and temporal expression of the first (Deluc, *et al.* 2008). Both of these classes are expressed in red and white grapevine cultivars, except for those regulating the expression of the UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) gene. The conversion from

anthocyanidins to anthocyanins by glycosylation, depends on its mediation (Boss, *et al.* 1996). In particular, the loss of pigmentation in white cultivars of *V. vinifera* L. is associated with multiallelic mutations in the regulatory genes *VvMYBA1* and *VvMYBA2*, which regulate anthocyanin biosynthesis (Kobayashi, *et al.* 2004). This section describes mostly the flavonoids present in white *V. vinifera* grapes (since the present research was focused exclusively on white varieties), with some reference to other species, anthocyanins are not described in detail.

The localization and organization of the enzymes responsible for phenylpropanoid and flavonoid biosynthesis were elucidated by immunolocalization experiments. These enzymes are structured in macromolecular complexes weakly associated with the endomembranes (endoplasmatic reticulum in particular) (Kutchan, 2005). The pigments, however, are accumulated in the vacuole or in the cell wall (Winkel-Shirley, 2001). However, in certain species, some of these enzymes are diversely located. To be precise: the aureusidin synthase, in the vacuole of *Anthirrihinum majus* (Ono, *et al.* 2006); a flavonoid 3'-hydroxylase in the tonoplast of the soybean immature seed coat (Toda, *et al.* 2012); and the flavonol synthase 1 (FLS1) in the *Arabidopsis thaliana* nuclei (Kuhn, *et al.* 2011). Interestingly, in *Arabidopsis* just a single copy of genes encodes for all the enzymes related to the central flavonoid metabolism, whereas the flavonol synthases (FLS) are encoded by six genes. Within those, just two of them (FLS1 and FLS3) are active (Owens, *et al*. 2008). Instead, more recent studies in *V. vinifera* demonstrated that within five *VvFLS* genes just the *VvFLS4* and *5* were transcriptionally active. The *VvFLS4* was proved to be responsive to UV-B in Sauvignon Blanc (Liu, *et al.* 2015). No clear evidence about flavonoid transport in plant is available to date. Two active transports (driven by ABC transporters and an antiporter) were found in *A. thaliana* (Frangne, *et al.* 2002, Klein, *et al.* 1996). In grape cell cultures four isoforms of glutathione S-transferases (GSTs) for anthocyanin transport to the vacuole, were reported (Zhang, *et al.* 2007). Moreover a bilitranslocase homologue carrier probably involved in flavonoid accumulation and transportation during berry development, was recently found in red (Braidot, *et al.* 2008) and white cultivars (Bertolini, *et al.* 2009).

From a biological standpoint, the flavonoids achieve several functions (Falcone Ferreyra, *et al.* 2012). It is known that they accumulate as a result of different kinds of stresses (Agati, *et al.* 2011, Kidd, *et al.* 2001, Haselgrove, *et al*. 2000, Cockell and Knowland 1999, Price, *et al.* 1995). The flavonoids achieve important roles in disease resistance mechanisms in plants (Winkel-Shirley 2001). One role is that they are a deterrent against plant herbivores and protect the tissues from pathogens (Falcone Ferreyra, *et al.* 2012). For example, flavan-3-ols and proanthocyanidins provide protection against fungal and bacterial pathogens, insect pests and larger herbivores (as reviewed by Dixon *et al.* 2005). Flavonoids can act as pre-infection toxins or as post-infection compounds. In the former case they are constitutive compounds, with concentrations high enough to defend the tissues against attack. In the latter case, when the plant detects elicitors during microbial attack, the phytoalexins are accumulated (Falcone Ferreyra, *et al*. 2012). In *V. vinifera* leaves flavonoid biosynthetic pathway genes were found to be modulated, as a result of virus infection (Gutha, *et al.* 2010). Flavonoids are also cellular mechanical support material (i.e. lignin and suberin). Another important role is their influence in plant allelopathy (Dey and Harborne 1997).

In some species (except in Arabidopsis) the implication of flavonols, especially the quercetin, in pollen germination and plant fertility (Mahajan, *et al.* 2011) was highlighted (Burbulis *et al.*, 1996). Moreover, according to the coevolution theory, the flavonoids, as secondary metabolites, were found to be important mediators for plant to insect and bird and mammal interactions (Bidart-Bouzat and Imeh-Nathaniel 2008). In flowers and fruits they contribute to the colour, which attracts the pollinators and seed dispersers. For instance, the yellow colour of the pollen depends on the different structures and combination of flavonoids (Zerback *et al*., 1989). This peculiarity is important because the reflection in the visible and UV spectra of these compounds could be detected by specific insects and animals, helping the pollination (Van Der Meer *et al.*, 1992). On the other hand, the flavonoid biosynthetic pathway induced by UV light was inhibited by pathogen-induced defence response in parsley, *Petroselium crispum* (Logemann and Hahlbrock 2002).

Flavonoid metabolite patterns of accumulation and profiling were also studied in several grape berry varieties for taxonomical purposes (Downey *et al*., 2003; Mattivi *et al*., 2006). The entire outcomes regarding the biological roles of flavonoid in biotic and abiotic stress response of the plants, allow us to hypothesize about their multi-functionality. A possible explanation might emerge analysing their chemical structure. In fact, the –OH group in $C_3$ of their skeleton could chelate metal ions, inhibiting the free radicals' formation and reducing the reducing the reactive oxygen species (ROS) once formed (Verdan *et al.,* 2011), for example.

Like all flavonoid compounds, the flavonols are phenylpropanoid derivatives, characterized by the three ring skeleton $C_6$-$C_3$-$C_6$. The $C_3$ side chain comes from the phenylalanine, and the $C_6$ ring derives from the acetyl-CoA groups from the polyketide pathway. Their 3-hydroxyflavone backbone has an unsaturated $C_3$ chain, with a double bond between carbons in position 2 and 3. The same biosynthetic pathway gives rise to condensed tannins and anthocyanins (red varieties) in grapevine (Stafford, 1990, Darne, 1993).

According to the biosynthetic pathway reported by Mattivi *et al.* (2006), six flavonols and their conjugated 3-O-glycoside forms can be found in grapes and wines: quercetin, myricetin, kaempferol, isorhamnetin, syringetin and laricitrin. Myricetin-type flavonols were found only at trace amounts in a couple of pink varieties. The delphinidin-like flavonols (myricetin, laricitrin and syringetin) were not at all detected in white grape varieties (Mattivi, *et al.* 2006, Castillo-Munoz, *et al.* 2009). This suggested that the gene coding for the enzyme F3'5' Hydroxylase is not expressed in white varieties (Mattivi, *et al.* 2006). The concentrations of free flavonol forms (after acid hydrolysis) were found to be three times higher in the skins of red grapes than in the white ones (Mattivi, *et al.* 2006, Cantos, *et al.* 2002). The quercetin-type flavonols were the main compounds in the flavonol profiles of both white (60-90 % of total flavonols) and red grapes (44 % of total flavonols). The highest flavonol concentrations in grape berries were found at flowering,

with a subsequent decrease while the berries' size increased. A second peak in their concentration was observed around one month post veraison (Downey, *et al*. 2003).

Flavonols accumulated as a result of different kinds of stress (Agati, *et al.* 2011, Haselgrove, *et al*. 2000, Cockell and Knowland 1999). As yellowish pigment they contribute to the colour of the pollen and the fruit (Van Der Meer, *et al.* 1992). In *V. vinifera* they are located in the vacuoles of the subepidermal skin cells (Cheynier and Rigaud 1986). Flavonols are present in grapes as glycosides (Ruberto *et al.*, 2007). They are important cofactors for colour enhancement and stabilization in wine (Roggero *et al*., 1997, Mistry *et al*., 1991, Scheffeldt and Hrazina 1998). Also, they are important because of their antioxidant activity in wine (Puertolas *et al*., 2010, De Beer *et al.*, 2005). Burda and Olezsek (2001) stated that within the flavonoids, only flavonols with a free hydroxyl group at carbon position 3 showed properties for scavenging reactive oxygen species. Other authors suggested also an implication of the double bond in position 2-3 in the *C* ring (responsible for electron delocalization) and the ortho-hydroxylation on the *B* ring of flavonoid molecule as condition of antioxidant and antiradical activities (Bors *et al.*, 1990, Bors *et al.*, 1997).

The flavan-3-ols and their polymers, the proanthocyanidins or condensed tannins, are accumulated in very significant quantities in *V. vinifera* berries (Kennedy *et al.*, 2000, Kennedy and Jones 2001). Indeed, they are considered to be the second most abundant flavonoid compounds (Hollman and Arts 2000).

The flavan-3-ols may occur in four isomeric structures (fig. 4). Nonetheless in nature only the 2, 3 trans (2R, 3S) isomer (catechin) and the 2, 3 cis (2R, 3S) isomer (epicatechin) are common. The 3- hydroxyl group can be esterified with gallic acid. In *V. vinifera* grapes the flavan-3-ols appearing as monomeric units are: (+)-catechin, (-)-epicatechin, (+)-epigallocatechin or (-)-epicatechin-3-O-gallate (Su and Singleton 1969). In the flavonoid pathway, all the intermediates up to leucoanthocyanidin, are of the 2, 3-trans stereochemistry; however, the most common extension unit in condensed tannin is 2, 3 cis epicatechin (Foo and Porter 1980). A possible role of flavan-3-ol monomers as

precursors in the condensation of polymeric proanthocyanidins was suggested (Douglas, 2006).

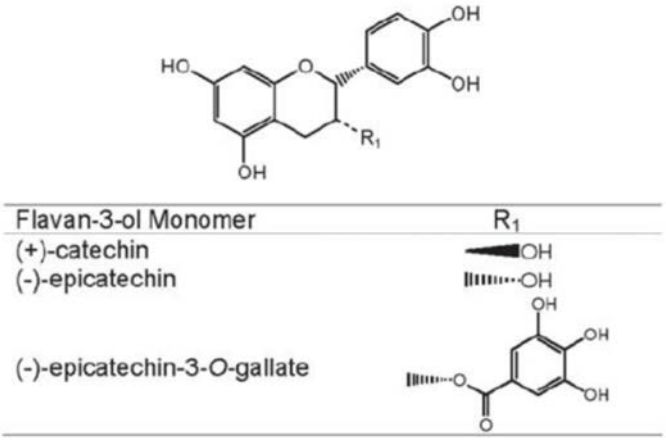


*Figure 4 Structure of grape flavan-3-ols (Kennedy, et al. 2006)*

When these subunits are organized in polymers, the carbon-carbon interflavan bond occurs mostly between the carbon 4 position and carbon 8 position of the adjacent subunit. This conformation leads to a final linear structure. Sometimes the bond can occur between the carbon 4 position and the carbon 6 position, leading to a final globular structure in branches (Prieur, *et al.* 1994, Souquet, *et al.* 1996).

Several hypotheses were suggested about the biosynthesis of the flavan-3-ols and their accumulation in the vacuole. The most common model is based on the evidence that a multi-enzymatic complex, which leads to the formation of the $C_6$- $C_3$- $C_6$ flavonoid skeleton, is bound to the endoplasmic reticulum (Wagner and Hrazdina 1984, Burbulis and Winkel-Shirley 1999, Kutchan, 2005). On this base the flavan-3-ols should accumulate in the vacuolar storage pool (Debeaujon, *et al.* 2001). Information on the mechanism of proanthocyanidin polymerization is inconsistent. There is little consensus on the polymerization process occurring in the vacuole, under acidic pH conditions (Zhao, *et al.* 2010, Davies, 1997, He, *et al.* 2008). Brillouet *et al*. (2013) suggested a model about the polymerization of monomers to tannins. In their opinion it is the differentiation of the chloroplast that leads to the condensed tannins. A direct polymerization inside the vacuole

would in fact lead to the denaturation of the vacuolar enzymes. During their microscopical studies they were able to describe different steps in this process. After a first unstacking and swelling of the grana thylakoids, some osmophilic materials emerged in the thylakoid lumen. Then the thylakoids began to pearl, generating the tannosomes. Those organules were then encapsulated in vesicles, generated from the budding off of the chloroplast and from the fusion of the plastid envelopes (Brillouet, *et al.* 2013).

The flavan-3-ols in the grape skin are located in the hypodermal cell layers (Adams, 2006). In the seeds they are in the soft parenchyma cells between the external hydrophobic cuticle and the inner lignified layers (hard seed coat) (Guerrero *et al.* 2009). In grape berries the tannin composition of the skin differs from that of the seeds, mostly in the proportional arrangement of their constitutive subunits. The seeds are richer in (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*o*-gallate (Su and Singleton, 1990). Whereas in the skin, there are additionally (+)-epigallocatechin (prodelphinidins) as extension subunits and, even if only in trace, there are the (-)-epicatechin-3-*o*-gallate and the (+)-epigallocatechin-3-*o*-gallate as a terminal or extension subunit (Escribano-Bailon, *et al.* 1995, Souquet, *et al.* 1996, Kennedy, *et al*. 2001, Bindon, *et al.* 2010b, Douglas, 2006). In both cases the catechin and epicatechin are the greatest extension subunits. A further distinguishing characteristic of the skin proanthocyanidins is the higher average mean degree of polymerization (mDP) (Bindon, *et al.* 2010b, Labarbe, *et al.* 1999). Furthermore, a lot of variability was reported about this latter topic during berry development (Kennedy, *et al.* 2001, Downey, *et al.* 2003a, Hanlin and Downey 2009). In grape berry skins, the tannins were found in multiple forms: free inside the vacuole, or bound to proteins to the internal face of the tonoplast, or linked to polysaccharides in the cell wall (Amrani-Joutei, *et al.* 1994). Tannins are mainly localized in the internal cell fraction, with a lower mDP than those of the cell wall (Gagne, *et al.* 2006).

The tannin subunits are synthesized in the skin after flowering, up to veraison (Kennedy, *et al*. 2001, Harbertson, *et al.* 2002, Downey, *et al*. 2003b). In the seeds the accumulation occurs at fruit set through veraison (Kennedy, *et al.* 2000, Downey, *et al*. 2006). The

hormone abscisic acid (ABA) is involved in the down regulation of the gene expression for the biosynthesis of the flavan-3-ol precursors to proanthocyanidins (Bogs, *et al.* 2005, Lacampagne, *et al*. 2010), which leads to a decline of skin proanthocyanidin content during the maturation (Lacampagne, *et al.* 2010). In point of fact, the flavan-3-ols and the anthocyanins share common steps in the flavonoid pathway (Bindon and Kennedy 2011, Kennedy, *et al.* 2001). These steps lead to their leucocyanidin (flavan-3, 4-diol) precursors (Stafford, 1990). However, the bibliography shows some discordances around the timing and the progression of proanthocyanidin accumulation in the skin. Indeed an increase, a decrease and a constant concentration were reported by different authors during ripening (Hanlin and Downey 2009, Fournand, *et al.* 2006, Canals, *et al*. 2005).

From a sensory standpoint, they have an impact on the complexity of the taste and mouth feel in the wine. The flavan-3-ols are compounds that potentially elicit bitterness (Yaminishi, 1990). The chiral difference between the (+)-catechin and the (-)-epicatechin change the perception and the duration of the bitterness feeling in the mouth. The (-)-epicatechin results as more involved in eliciting the bitter sensation, because the conformation of the *C* ring is planar and, thus, more lipophilic (Haslam, 1982, Peleg, *et al.* 1999), while the substitution with gallic acid increases the astringency (Brossaud, *et al.* 2001). During the maturation the bitterness sensation decreases because the degree of flavan-3-ol polymerization increases, leading to a higher perception of astringency (Cheynier, *et al.* 2006). The proanthocyanidins cause astringency (Dixon, *et al.* 2005). Higher tannin concentrations lead to higher astringency. Nevertheless, some authors found that the degree of polymerisation was inversely correlated to astringency (Souquet, *et al.* 1996). Tannin extractability decreases during seed maturation, as a consequence of their polymerization. It was demonstrated that the amount of proteins precipitated by tannins, increased with increasing polymer size (Hagerman, *et al.* 1998, Harbertson, *et al.* 2014). This occurs because additional ortho di-hydroxy groups in the tannins provide more sites for hydrogen bonding with proteins. Also, a mixture of different-sized tannins precipitates proteins with an additive effect (Harbertson, *et al*. 2014).

## 1.3 Abiotic stress effects

Over the last years, the most studied abiotic cues affecting the biochemical parameters related to berry ripening were light conditions (Downey, *et al.* 2004, Spayd, *et al.* 2002, Keller, *et al.* 1998, Keller and Torres-Martinez 2004), nutrient availability (Keller, *et al.* 1998), water status (Lovisolo and Schubert 1998, Matthews and Anderson 1998, Keller 2005, Kennedy, *et al.* 2002) and temperature (Spayd, *et al.* 2002, Tarara, *et al.* 2008)

Flavonoids are involved in UV scavenging (Winkel-Shirley, 2001). Several studies focused on understanding whenever the UV-B radiation might induce the accumulation of flavonoids in plant tissues (Cockell and Knowland 1999, Price, *et al.* 1995, Haselgrove, *et al.* 2000). Recently flavonol biosynthesis and concentration were demonstrated to be light dependent (Downey, *et al.* 2003b, Price, *et al.* 1995). Additionally, quercetin glycoside accumulation was found to be strongly modulated by UV-B (Gotz, *et al.* 2010, Agati, *et al.* 2011). Furthermore, an enhancement in the biosynthesis of this compound was observed in sun exposed leaves, with or without UV radiation presence (Agati, *et al.* 2011). Shiraz berries grown under shaded conditions showed lower flavonol concentrations at harvest time (Downey, *et al.* 2004). This result was explained as mostly due to a reduced biosynthesis, rather than degradation during ripening. Sun exposed berries showed higher content in flavonols glucosides than the shaded ones (Price, *et al.* 1995). More recently, other authors found a negligible level of flavonols in both leaves and berry skins when they were not exposed to light. (Downey, *et al.* 2003a). Subsequent exposure of those tissues to sunlight resulted in a rapid increase in those compound accumulations and in the expression of the gene encoding flavonol synthase (Downey, *et al*. 2003b). For the same authors there was no observable effect of bunch exposure on either the proanthocyanidin content or composition in seeds and in the skins at harvest. Changing in cluster microclimate by leaf removal, increased the concentration of all flavonols, but not that of flavanol (Friedel, *et al.* 2015). In other studies the effect of light on flavanol accumulation was shown when the leaf removal was applied right at the beginning of flowering (Koyama, *et al*. 2012). Indeed, even if an increase in flavanol

levels occurs during maturation, their synthesis is no longer subject to light influence (Friedel, *et al*. 2015).

Among the factors that could affect the flavonoid composition of plant tissues, the nutrient availability surely plays a key role (Keller, *et al.* 1998). For example, it was demonstrated that an excess in nitrogen fertilizer levels determined a decrease of grape berry color (Keller and Hrazdina 1998). Also the vine vigor affected the tannin content and composition of grape skins (Cortell, *et al.* 2005). Indeed, changes in proanthocyanidin level and composition were found. A higher concentration and an increase in the average size of polymers, associated with an increase in the proportion of epigallocatechin subunits, were observed when the vine vigor decreased (Cortell, *et al.* 2005). Moreover it was uncertain whether these changes were due just to the difference in vine vigor or also to an indirect effect caused by changes in canopy architecture which resulted in different bunch exposures. Agati *et al*. (2011) described higher flavonol levels in case of root zone salinity. This latter condition, in concomitance with UV-B radiation, determined a depletion in ascorbate peroxidase and a parallel accumulation of quercetin-3-O-glycosides (Fini *et al.,* 2011). Other authors (Kidd *et al.,* 2001) found high levels of quercetin and catechin exudates in leaves of maize exposed to aluminium. Also quercetin and kaempferol were found to be implicated in local auxin accumulation in plants under poor growth conditions. Apparently these flavonols inhibited the transportation of this hormone, which is involved in stress response (Peer and Murphy 2007, Lewis *et al.,* 2001). Some scientists speculate that flavonoid biosynthesis may be upregulated as result of serious alterations in cellular ROS/REDOX homeostasis, as a consequence of the decrease or inactivation of the enzymes involved in primary antioxidant defences (Taylor and Grotewold 2005). On the other hand, the lack of flavonoids was shown to determine nodulation deficiency in transgenic plants (Wasson *et al.,* 2006). The deficiency of flavonan-3-ols and flavonols led to parthenocarpy in transgenic tomatoes (Schijlen *et al.,* 2007). The silencing of FLS in tobacco led to an increase of seedless fruits and lower levels of flavonols and anthocyanidins, whether an increase in flavan-3-ols was demonstrated (Mahajan *et al.,* 2011).

Even the role of water stress was shown to affect the tannin content. Some authors suggested that while an excess in water application decreased the tannin content (Kennedy *et al.*, 2000), a water deficit had little or no effect on tannin or anthocyanin accumulation in the grape berry (Kennedy *et al.*, 2002). 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.

The temperature had no effect on total flavonol concentrations, whereas it significantly reduced the level of anthocyanins, through degradation and/or inhibition of synthesis (Kliewer and Torres 1972, Spayd *et al.*, 2002).

## 1.4 Introduction to destructive and non-destructive pigment and flavonoid determination

In agriculture various technologies using remote and/or proximal sensing acquisitions have been developed and used over the last years. The power of these new techniques comes from the multiple opportunities generated by their usage. For example, they provide noninvasive information at high resolution and multiple high density observations on the same sample. Moreover, costs are usually reasonable with a consistent reduction in terms of investigation time (Abbot 1999, Gitelson, *et al.* 2002). Spectroscopic and imaging techniques were studied with different goals, such as to describe fruit physiology and quality (Merzlyak, *et al.* 1998), to detect symptomatic and asymptomatic plant diseases and stresses (Bellow, *et al.* 2012) and to evaluate the yield, the ripeness and the spatial and temporal variability (Bramley and Hamilton 2004, Baluja, *et al.* 2011). Furthermore, nondestructive phenotyping and studies regarding the physiological disorders generated by sunburn of grapevine berries were suggested (Rustioni, *et al*. 2014).

All these applications are possible because the spectral signature of a tissue depends on the combination within its chemical and structural composition (Butler 1960, Gitelson, Gritz and Merzlyak 2003, Solovchenko, 2003). Conventionally pigment determination

requires tissue extraction, but nowadays, nondestructive optical methods based on spectral indices and formulas have been developed and are in use (Gitelson, *et al.* 2002). Nevertheless, to estimate pigments it is necessary to find spectral bands where the reflectance is maximally sensitive to the compound of interest and minimally sensitive to others (Solovchenko, *et al*. 2010). For example, the chlorophyll shows two intense absorption bands in the blue (400-500 nm) and red (600-700 nm) regions, while carotenoids and xanthophylls mainly absorb into the green-blue wavelengths. Normalized reciprocal reflectance (R) at specific wavelengths, was demonstrated to correlate with pigment concentrations in leaves (Gitelson, *et al.* 2003) and apple fruits (Merzlyak, *et al.* 2003b). For instance, the reflectance ratios $R_{800}/R_{700}$ and $R_{800}/R_{640}$ were found to be proportional to total chlorophyll, ranging from 0.4-11 nmol/cm$^2$ ($r^2$>0.93) (Merzlyak, *et al.* 2003b). Previously, other authors proposed $R_{675}$ to estimate skin chlorophyll content in apples (Knee, 1980) and $\log R_{680}$ for chlorophyll estimation, ripening and internal quality assessment in orange (Chuma, 1981). In the reflectance spectrum a distinct minimum near 678 nm was attributed to chlorophyll *a*, while a shoulder near 650 nm to chlorophyll *b* (Merzlyak, *et al.* 2003b). Also, a relationship between chlorophyll and carotenoid concentration was demonstrated, through ripening. In fruit with healthy tissues, an increase in the red reflectance, was accompanied by a slight increase in the blue range, characteristic of chlorophyll breakdown and carotenoid retention during leaf senescence and fruit ripening (Merzlyak, *et al.* 1998). A strong overlapping of light absorption by chlorophylls, carotenoids and pigments responsible for brownish coloration was found in apple fruit (Chivkunova, *et al.* 2001). Different studies demonstrated optical changes in concomitance with browning tissues, no matter if it was induced by heating (McClure, 1975), wounding (Merzlyak, 1990) and/ or ageing (Merzlyak, 1999). In fact, a decrease especially in the green, was detected during browning (Chivkunova, *et al.* 2001). Thus this shift of chlorophyll absorption maximum to shorter wavelengths was proposed as indicator of tissue browning appearance in apple (Chivkunova, *et al.* 2001, Solovchenko, *et al*. 2010).

Nonetheless, depending on the final goal, it is often necessary to use destructive techniques for pigment and flavonoid determination. In fact non-invasive tools allowed a good estimation of a certain compound, but a calibration with wet chemistry methods is always necessary. One of the most used techniques allowing a qualitative and quantitative determination is the liquid chromatography. It is especially used when a separation of the compounds in a sample solution is required (Kammerer, *et al*. 2004). Indeed, it could be that a mixture is too complex for a direct analytical measurement (e.g. spectroscopy), especially when concentration is quite low (e.g. depending on the phenological stage). The concept behind liquid chromatography is quite intuitive: all the components of a mixture interact differently with the environment, when all of them are put under the same conditions (Bidlingmeyer, 1992).

The high performance liquid chromatography (HPLC) process is quite simple to explain, but needs time to get used with. First the samples must be extracted in a suitable solvent. The extracts (HPLC purity grade) are pooled in an auto-sampler vial (pre-labelled) and automatically injected into one end of the selected column, by a precise volume injector. A pump delivers the mobile phase through the column. The volume injected is carried by a continuous flow of the mobile phase eluents. The separation of the molecules takes place in the column, which contains the stationary phase (tightly packed chromatographic particles). It occurs because each molecule interacts with the stationary phase in a continuous manner. Consequently, some components of the mixture (extract plus mobile phase) migrate through the column more slowly than the others. As the sample components emerge from the column, a diode-array detector (DAD) transmits a signal to a recording device. The result is a chromatogram, in which the compounds are indicated as peaks, in function of their elution times. The identification of the single compounds is carried out using specific standards and comparing the elution times and the maximum absorption peak and the spectra with those referenced in the literature. This technique is moderately to quite expensive, due to labour intensive and time consuming sample preparation by trained personnel required for careful handling of samples, as well as computer necessary for data analysis.

## 1.5 Ph.D. project objectives

Fruit quality involves sensorial properties, nutritive values, chemical composition, structural and mechanical features and absence of defects (Abbot, 1999). Chlorophyll, carotenoid, anthocyanin (in red grapes) and flavonol contents and their proportions, determine fruit color and appearance (Saure, 1990, Abbot, 1999) and serve as markers of quality.

Considerable research was done on grapevine canopy management (Smart 1985) to provide the right microclimate to the bunches during ripening. Indeed, the sunlight exposure and temperature may affect berry composition and metabolism, also determining browning appearance (Spayd *et al.* 2002). The browning of berry skin is a symptom of sunburn, but could also be a manifestation of physical damage, physiological disorders, senescence, diseases and hypersensitive reaction to pathogenic microorganisms (Delalieux, 2007). In general, this process is related to the loss of cell compartmentation and the oxidation of polyphenolic compounds by polyphenol oxidase, resulting in polymers (Vaughn, 1984). Phenolic oxidation products are difficult to measure because of their structural heterogeneity. Nevertheless, an indirect analysis of the variation in photosynthetic pigments and flavonoid compounds under radiative stress might be a possible indicator for the estimation of damage severity. Indeed, flavonoids also play a major role in light stress response (Kolb *et al*., 2003).

The background hypothesis of the present work was to understand if a decrease or increase in photosynthetic pigments and flavonoids, could be good descriptors for berry physiological response to excessive sunlight and temperature exposure, as well as good estimators of varietal tolerance and/or susceptibility to radiative stress. The effect of water stress was also considered, because the shoot growth is extremely responsive to the irrigation techniques (Keller *et al*., 2008), thus may affect the bunch microclimate. Plus, a part of the research was conducted in the Columbia Valley, Washington State, USA, where the extremely low annual precipitation (about 200 nm), prohibits grape-growing, if irrigation is not applied (Keller *et al*., 2008).

In previous research, non-destructive reflectance was used to study the appearance of oxidation polymers as result of sunburn in white grape berries (Rustioni *et al.*, 2014). A decrease of the reflectance spectrum in the blue-green was typical of berry exposure to excess of light. Also, a possible involvement of photosynthetic pigments was found, with a positive correlation between chlorophyll content and browning appearance (Rustioni *et al*., 2014).

Nonetheless, chlorophyll, carotenoid and xanthophyll extractions are difficult to manage, since chlorophyll pheophytinization, and cis/trans isomerization of carotenoids could easily occur while processing the samples (Van den Berg *et al.*, 2000, Kamffer *et al.*, 2010). One of the goals in my PhD research was to facilitate the study of the photo-oxidative roles of photosynthetic pigments, by developing new reflectance indices for their non-invasive assessment in grape berries. Also, the effects of two different berry exposures timing on sunburn susceptibility and/or tolerance, by leaf removal, was tested. This part of my investigation was conducted in Italy, at the University of Milan and is described in chapter 2.

The flavonols and flavanols evaluation in Chardonnay and Riesling berries in response to sunlight exposure, under different irrigation regimes, and the relation of water stress to sunburn appearance, were the main topics of the second part of my research that was conducted at the Washington State University, Irrigated Agricultural Research and Extension Center (Prosser, Washington State) and are described in chapter 3.

# Chapter 2 Italian experimentation

## 2.1 Materials and methods

### *2.1.1. Plant material and growth conditions*

The *Vitis vinifera* L. accessions used in this 2013 study were all sourced from the same germplasm collection vineyard, located in the Regional Research Station of Riccagioia, (Lombardy region, Northern Italy). The vineyard is located on a hilly terrace with a slight eastward exposition (longitude 9°05', latitude 44°58', elevation 144 m a.s.l.). The soil is clayey: Udic Paleustalfs fine silty, mixed, superactive, mesic (Soil Survey Staff 1999). According to Koeppen' classification, the climate zone is mesothermal with transitional characteristics between Oceanic (Cfb) and Mediterranean (Csa) types, (Strahler, 2013). The annual rainfall range from 600 to 800 mm with two maxima (in spring and fall). The minimum winter and summer temperatures are 0.7 and 22.4°C respectively, with an annual average of 11.7°C (Rustioni, *et al.* 2013). Plants are spaced by 2.5 m × 1 m, with about 4,000 plants/hectare. The trellis system is a 10–12 buds cane Guyot. The space within the row was treated twice per year with glyphosate herbicide.

### *2.1.2 Experimental design and sample collection*

Sixteen white wine and table grape cultivars were selected: Chardonnay, Italia, Matilde, Moscato Giallo (syn. Goldmuskateller), Pedro Ximenez, Perle of Csaba, Pizzutello (syn. Cornichon Blanc), Regina (syn. Afuz Ali), Regina dei Vigneti (syn. Muscat Queen of Vineyards), Ribolla Gialla, Riesling, Rkatsiteli, Sultanina, Verdeho Blanco, Verdicchio, Zibibbo (syn. Muscat of Alexandria). One cluster for each accession was sampled at different phenological stages, following the BBCH scale (Lorenz, *et al.* 1995): pre-veraison (77 BBCH "berries beginning to touch"), veraison (81 BBCH "beginning of ripening: berries begin to soft"), and harvest time (89 BBCH "berries ripe for harvest"). For each accession the investigations were conducted on four berries. On each one, the weight, the axial and abaxial diameters and the skin chlorophyll and carotenoid (plus xanthophyll) concentrations by destructive quantifications were measured. The reflectance spectra in four different positions were measured after epicuticular wax

removal (Rustioni, *et al.* 2012). On Chardonnay and Riesling the effects of different defoliation timing on sunburn susceptibility and/or tolerance, were also studied at 77, 81 and 89 BBCH. For each of these two cultivars, two additional clusters were completely exposed to direct sunlight, with defoliation, respectively at 12.30 p.m. and 6.30 p.m. solar time, and the epicuticular waxes were mechanically removed from the SW side facing the sun, to facilitate the sunburning symptoms appearance. The clusters were harvested the day after at 12.30 p.m. The most symptomatic twenty berries were chosen and used as replicates, for the additional reflectance spectra analysis, to describe the browning of the skins.

### *2.1.3 Berries optical properties detection*

Overall 768 reflectance spectra were obtained using a spectrometer Jaz System (Ocean Optics, B.V.), completed with a Channel with a DPU Module and ILX511b Detector, OFLV-3 Filter, L2 Lens and 50 µm Slit as installed options. A reflection probe QR600-7-VIS125, consisting of a tight bundle of 7 optical fibers (600 µm in diameter), in a stainless steel ferrule (6 illumination fibres around 1 read fibre), was coupled to the spectrophotometer. The instrument was set up with a NIR/Vis light source 4095 power setting (500 hours of lamp life), and an integration time was corrected in order to give the best percentage reflection during the calibration. Collected spectra ranged between 340 and 1025 nm, and they had a wavelength interval of about 0.3 nm. In this work, just the visible spectral changes (450-750 nm) at wavelength of 1 nm interval are present and discussed. Each spectrum was set up to be the average of 20 spectra, which were directly calculated by the instrument. The spectra were measured in percentage of reflectance (%R). A calibration with a reference spectrum was obtained by taking the light source on and by using the blank WS-1 Diffuse Reflectance Standard made of PTFE, (Ocean Optics, B. V.). Also a dark spectrum was taken with the light path blocked.

### *2.1.4 Reflectance spectra elaborations*

The relationship between reflectance signal and chromophore content is nonlinear. Thus the reflectance spectra were converted to approximate to the (quasi) almost linear

relationship between pigment content and optical reflectance-based indices, using reciprocal reflectance (1/R) spectra.

Each single spectrum was used to validate and calibrate existents reflectance indices for the carotenoid and chlorophyll estimations.

In particular, concerning chlorophylls:

$Chl = (R_{800}/R_{678})$ (Merzlyak, *et al.* 2003b)

$Cl_1 = (1/R_{700}-1/R_{800})*R_{800}$ (Gitelson, *et al.* 2003)

$Cl_2 = (1/R_{640}-1/R_{800})*R_{800}$ (Gitelson, *et al.* 2003).

While the indices considered for the carotenoid estimation were:

$CRI_1 = (1/R_{520}-1/R_{700})*R_{800}$ (Merzlyak, *et al.* 2003b);

$CRI_2 = (1/R_{520}-1/R_{550})*R_{800}$ (Merzlyak, *et al.* 2003b).

All these indices were proposed on the base of the reflectance of leaves and apple fruit.

Thus the visible range of the spectrum was also entirely considered (450-750 nm) with the aim to develop new, more performant and accurate indices able to better distinguish the photosynthetic pigments in grape berry skin.

To evaluate the sunburn appearance, it was used the browning intensity index:

$BI= (100\ R_{e678}/\ R_{e490})-(100\ R_{s678}/\ R_{s490})$ (Rustioni, *et al.* 2014).

Where: *e* = exposed berries and *s* = shaded berries (control)

### *2.1.5 Photosynthetic pigment analysis*

HPLC degree ethanol (95%) and calcium carbonate were all Sigma. The berries were weighed and the diameter was measured. Pigment content was determined from the same berry samples used for reflectance measurements. Four berries for each accession were

squeezed to separate the exocarp from the pulp. Each exocarp was ground under liquid nitrogen, in a mortar with pestle, to a fine powder; and 1.5 mg of $CaCO_3$ salt was added during tissue grinding to prevent the formation of pheophytins during chlorophylls extraction and to neutralize acids in tissue samples to avoid cis/trans isomeration when extracting carotenoids (Van den Berg, *et al.* 2000, Kamffer, *et al.* 2010). The ground powder, combined with three washings of the pestle and mortar (each of about 1.5 mL of ethanol 95%), was poured into a Falcon tube and extracted by a total of 5 mL of solvent (ethanol 95%).

All procedures were carried out in subdued light to minimized light-associated degradation of chlorophylls and carotenoids. In fact, approximately a 30% increase in the extraction of lutein, zeaxanthin, and *β*-carotene was found in tissue under subdued light conditions, compared to that in normal laboratory light conditions during extraction from both green and red berry tissues (Kamffer, *et al.* 2010).

After 20 minutes the homogenate was centrifuged at 10,000 rpm for 10 minutes to make the extract fully transparent. Afterward the supernatants were poured into a new Falcon tube and adjusted to a final volume of 5 mL with Ethanol 95%. The spectrum was recorded at 470 nm, 648.6 nm and 664.2 nm in a Jasco model 7800 spectrophotometer. Specific absorption coefficient and formulas were used to calculate chlorophyll *a*, *b*, *a+b* and carotenoid + xanthophyll concentrations (Lichtenthaler, 1987).

$C_a = 13{,}36 * A_{664,2} - 5{,}19 * A_{648,6}$

$C_b = 27.43 * A_{648.6} - 8.12 * A_{664.2}$

$C_{(a+b)} = 5.24 * A_{664.2} + 22.24 * A_{648.6}$

$C_{(x+c)} = (1000 * A_{470} - 2.13 * C_a - 97.64 * C_b)/209$

Where:

$C_a$ stands for chlorophyll a; $C_b$ stands for chlorophyll b; $C_{(a+b)}$ stands for total chlorophyll content; $C_{(x+c)}$ represent carotenoid content, including xanthophyll and *β*-carotene.

The berry surface was calculated by the measured diameters. Berries were compared to prolate or oblate spheroids (a=b>c in oblate ellipsoid of revolution and a=b<c in prolate ellipsoid of revolution, where a, b and c are the semi-principal axes length). The surfaces (S) were calculated as follows:

$$S\ (oblate) \approx 2\pi\ [a^2 + (c^2 * arctanh\ (sin\ (æ))/sin\ (æ))$$

$$S\ (prolate) \approx 2\pi\ [c^2 + ac * æ/sin\ (æ))$$

$$æ = arcos\ (c/a)$$

Pigment concentrations were converted into μg/cm$^2$, to be compared with the optical properties of the berry surface.

### *2.1.6 Theoretical absorbance bands calculation*

The theoretical absorbance bands in fig. 1, were calculated as Gaussian functions. The absorption maxima were fixed at the experimental wavelength peaks, and the intensity was calculated considering the molar extinction coefficient proposed by Lichtenthaler (1987). Each obtained Gaussian was then multiplied by the average experimental quantification of chlorophyll *a* and *b*, respectively. In this way, it was possible to hypothesize the absorption contribution of each pigment in the red spectra region.

### *2.1.7 Statistical analysis*

Regression models were used to compare the concentration (μg/cm$^2$) of the photosynthetic pigment with the average of the four spectra measured on each berry. The software used was SPSS© (PASW Statistics 21 version, SPSS Inc. Chicago, Illinois).

## 2.2 Results

Chlorophyll *a* concentration, expressed as mean of all varieties, changed from 2.1 µg/cm$^2$ at 77 BBCH, to 0.97 µg/cm$^2$ at 89 BBCH (fig. 5), while chlorophyll *b* was less concentrated. In pre-veraison the mean concentration was 1.4 µg/cm$^2$, later to drop to about 0.6 µg/cm$^2$. A general decrease in total chlorophylls was visible from pre-veraison 3.5 µg/cm$^2$ to harvest 1.5 µg/cm$^2$, carotenoids also decreased during berry development. At 77 BBCH the average concentration was 0.58 µg/cm$^2$, at stage 81 BBCH it was 0.47 µg/cm$^2$ and at 89 BBCH it decreased until 0.38 µg/cm$^2$.

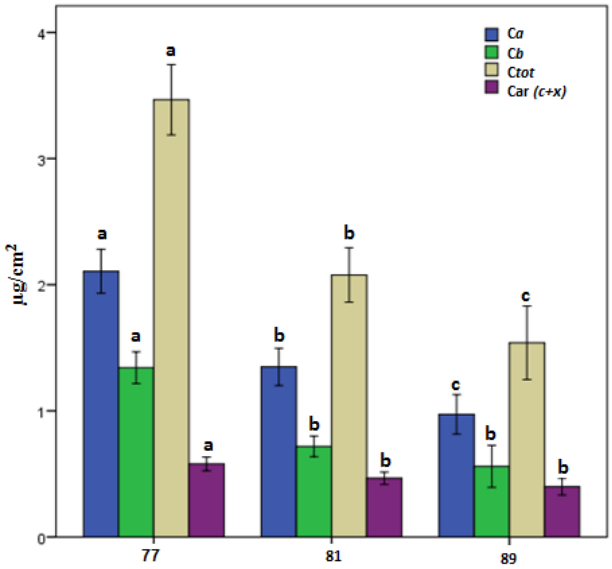


*Figure 5 Pigment content variation for all varieties, during the three different phenological stages (77, 81, 89 BBCH), with standard errors. The bars represent respectively chlorophyll a (µg/cm²), chlorophyll b (µg/cm²), total chlorophyll (µg/cm²) and carotenoids plus xanthophylls (µg/cm²). The letters represent the significance referred to the single compound.*

All reflectance (R) spectra collected were converted into their reciprocal (1/R), and then a normalization at 800 nm was applied to facilitate the data comparison. $R_{800}/R_x$ spectra were calculated to analyse the maximum peaks of both chlorophylls *a* and *b*. A particular detail of the spectral range 600-750 nm (obtained by the average values of all the 768 spectra) is shown in figure 6 (dark area).

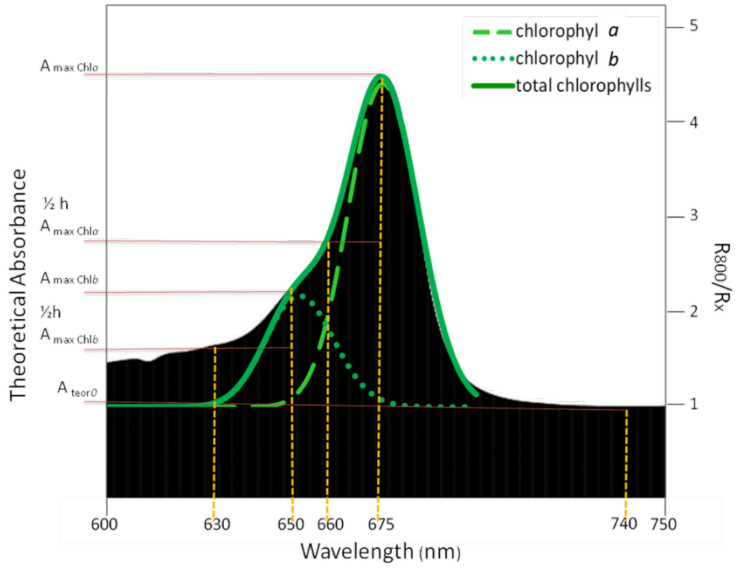


*Figure 6 Chlorophyll indices explication. The dark area represents the average (768 measurements) reciprocal reflectance multiplied for the 800 nm reflectance value. The green lines draw the expected absorption bands of the pigments. The wavelengths of interest for the half height are indicated.*

$R_{800}/R_{675}$ was found to be the maximum reflectance edge of chlorophyll *a*, while for chlorophyll *b* it was $R_{800}/R_{650}$. The absorbance at 660 nm and 630 nm were the best descriptors for the chlorophylls *a* and *b* reflectance at half-height of the Gaussian' curve. The differences between these values should then be related with the pigment concentrations. Thus, $(R_{800}/R_{675})$-$(R_{800}/R_{660})$ was calculated for chlorophyll *a* and $(R_{800}/R_{650})$-$(R_{800}/R_{630})$ for chlorophyll *b*. Chlorophyll indices were calculated as follows:

$$C_aI = log\ [(R_{800}/R_{675})-(R_{800}/R_{660})]$$

$$C_bI = log\ [(R_{800}/R_{650})-(R_{800}/R_{630})]$$

$$C_{tot}I = C_aI + C_bI$$

$C_aI$: chlorophyll *a* index; $C_bI$: chlorophyll *b* index; $C_{tot}I$: total chlorophylls index; $R_x$: reflectance at x wavelength.

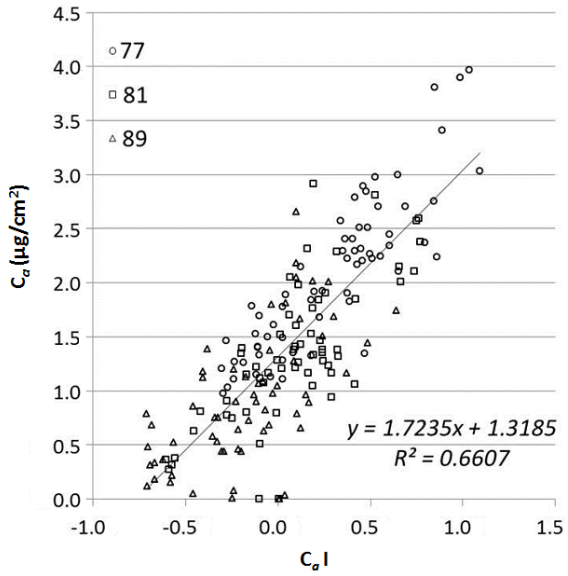


*Figure 7 Correlations between the Chlorophyll a Index $C_aI$ (average of 4 replicates/berry) and the measured chlorophyll a concentration (192 berries in total).*

Figure 7 describes the comparison between $C_aI$ and the measured chlorophyll *a* content (µg/cm$^2$) during pre-veraison, veraison and harvest. A linear model was able to define this relationship, with an $r^2$=0.66. It was possible to estimate the chlorophyll concentration by the formula:

$$C_aE = 1.7235*C_aI + 1.3185$$

$C_aI$: chlorophyll *a* index; $C_aE$: estimated chlorophyll *a* concentration.

The same approach allowed to estimate the chlorophyll *b* (fig.8).

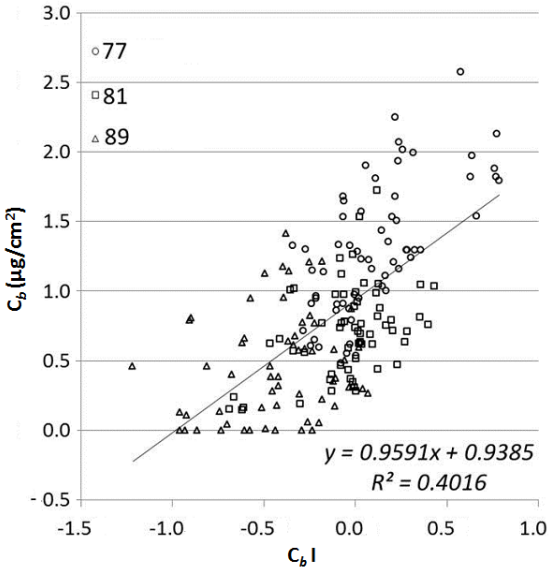


*Figure 8 Correlations between the Chlorophyll b Index $C_bI$ (average of 4 replicates/berry) and the measured chlorophyll b concentration (192 berries in total).*

$C_bE = 0.9591*C_bI + 0.9385$

$C_b$I: chlorophyll *b* index; $C_b$E: estimated chlorophyll *b* concentration.

The sum of $C_a$I and $C_b$I, provided an estimation of the total chlorophyll contents $C_{tot}$I $r^2$=0.63 (fig. 9).

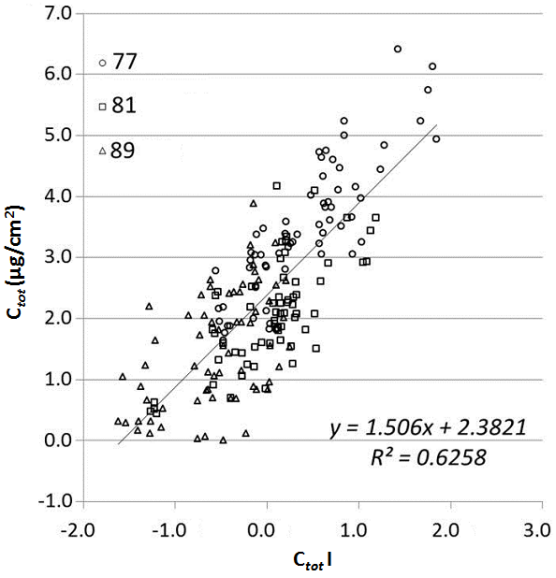


*Figure 9 Correlations between the total chlorophyll Index $C_{tot}I$ (average of 4 replicates/berry) and the measured total chlorophyll concentration (192 berries in total).*

Other indices proposed by the literature were also validated (tab. 1).

*Table 1 Correlations between pigments concentrations (µg/cm²) and reflectance indices.*

| References | Indexes | Formulas | Pigments | $r^2$ |
|---|---|---|---|---|
| Merzlyak, *et al.* 2003b | Chl Index | $R_{800}/R_{678}$ | Total chlorophylls | 0.594 |
| Gitelson, *et al.* 2003 | Cl 1 | $(1/R_{700}-1/R_{800})*R_{800}$ | Total chlorophylls | 0.214 |
| Gitelson, *et al.* 2003 | Cl 2 | $(1/R_{640}-1/R_{800})*R_{800}$ | Total chlorophylls | 0.482 |
| Merzlyak, *et al.*2003b | CRI 1 | $(1/R_{520}-1/R_{700})*R_{800}$ | Carotenoids | 0.051 |
| Merzlyak, *et al.* 2003b | CRI 2 | $(1/R_{520}-1/R_{550})*R_{800}$ | Carotenoids | 0.021 |
| present work | Car Index | $R_{800}/R_{530}$ | Carotenoids | 0.073 |
| present work | CarI | $a*C_aI+b$ | Carotenoids | 0.726 |
| present work | $C_aI$ | $\log[(R_{800}/R_{675})-(R_{800}/R_{660})]$ | Chlorophyll *a* | 0.661 |
| present work | $C_bI$ | $\log[(R_{800}/R_{650})-(R_{800}/R_{630})]$ | Chlorophyll *b* | 0.402 |
| present work | $C_{tot}I$ | $C_aI + C_bI$ | Total chlorophylls | 0.626 |

Among the other proposed indices, one proposed by Merzlyak, *et al.* (2003b), was the most sensitive for total chlorophyll estimation ($r^2$=0.59). The others were unable to provide high correlations between reflectance spectra and pigment contents.

For carotenoid estimation different indices were verified. For example, an index $R_{800}/R_{530}$ based on the same model of Merzlyak, *et al.* (2003b) was calculated, but no correlation was achieved ($r^2$ = 0.07). Thus, the relationship between carotenoids and chlorophyll *a* was studied. The correlation between these two pigments resulted very high. At the BBCH 77, 81 and 89 $r^2$ were 0.73, 0.70 and 0.52 respectively (fig. 10: 1, 2 and 3 respectively).

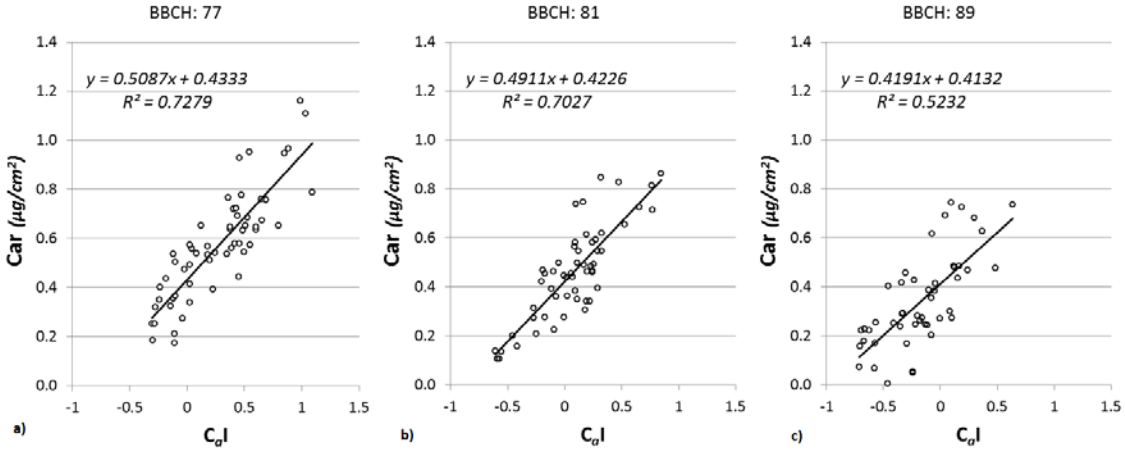


*Figure 10 Correlation between the Chlorophyll a Index $C_aI$ (average of 4 replications/berry) and the carotenoid quantification (192 berries in total) during berry development (10a: BBCH 77; 10b: BBCH 81; 10c: BBCH 89). During ripening, the regression line becomes flatter, indicating a decrease in the chlorophyll/carotenoid ratio.*

On this base it was possible to gain a carotenoid index (*CarI*), calibrated by the regression curve:

$$CarI = a * C_aI + b$$

*CarI*: carotenoid index; $C_aI$: Chlorophyll *a* Index; a: slope; b: y-intercept.

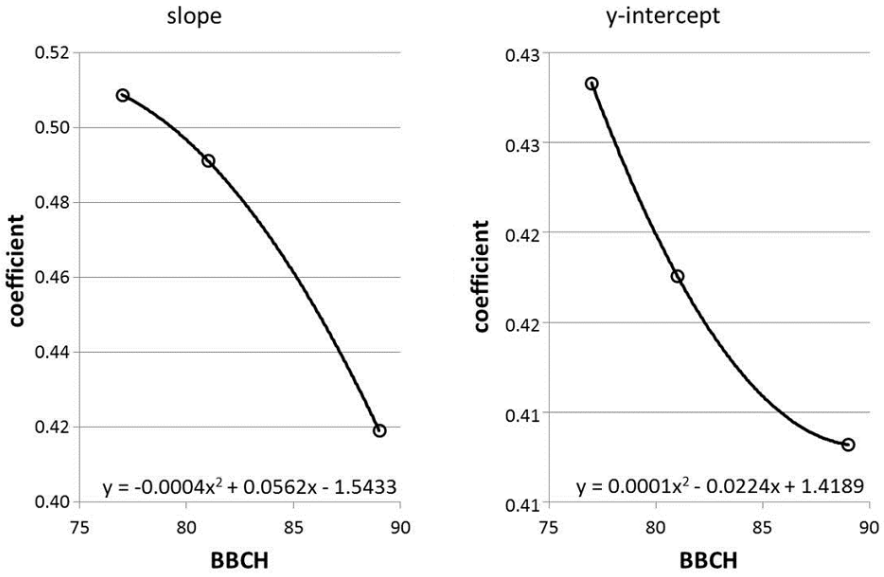


*Figure 11 Carotenoid index coefficients. Changes in slope and y-intercept during berry development. Dots represent the slopes and y-intercepts shown in figure 10. By using the regression curve it is possible to calculate the best coefficient at each BBCH phenological stage.*

Figure 11 shows the variation of index coefficients during the phenological stages. By the found regressions it was possible to calculate the best coefficients (slope and y-intercepts) at each BBCH phenophase, in the range from 77 (pre-veraison: "berries beginning to touch") to 89 (ripening: "berries ripe for harvest"):

$$a = -0.0004*PP_{BBCH}^{2} + 0.0562PP_{BBCH} - 1.5433$$

$$b = 0.0001*PP_{BBCH}^{2} + 0.0224PP_{BBCH} - 1.4189$$

*a*: slope; *b*: y-intercept; $PP_{BBCH}$: BBCH phenophase.

Using these phenological dependent coefficients, it was possible to achieve a carotenoid index (*CarI*) based on the $C_a$I.

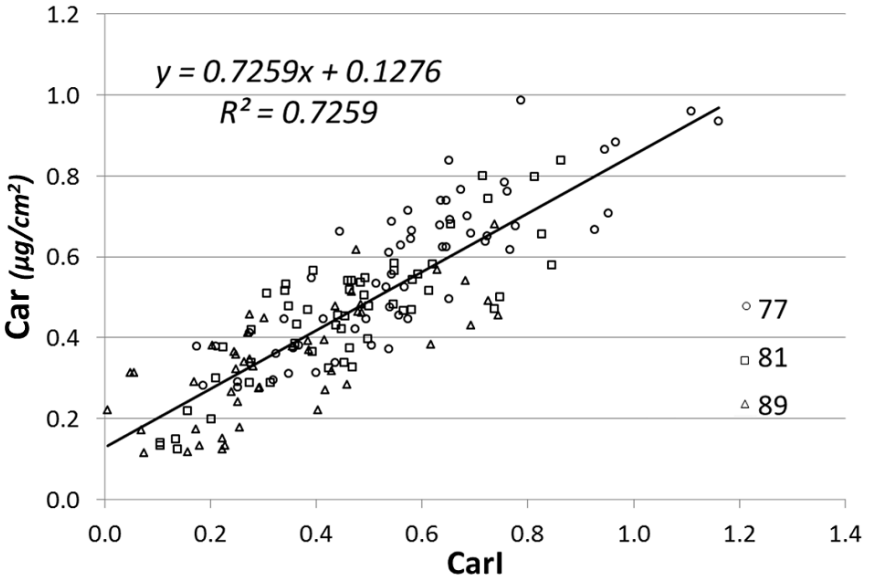


*Figure 12 Correlation between the Carotenoid Index CarI (average of 4 replicates/berry) and the carotenoid concentration (192 berries in total).*

Figure 12 shows the correlation between *CarI* and carotenoid concentrations $r^2 = 0.73$, quantified by wet chemistry.

During the analysis of the same reflectance spectra, a possible way was studied to understand white berry skin-color variation over the maturation, considering the photosynthetic pigments. Just three of the measured cultivars were discussed: Ribolla Gialla, Pedro Ximenez and Moscato d'Alessandria.

Figure 13a shows a particular (450- 750 nm) of the reflectance spectra (R %) for three accessions during the three different phenological stages, 77, 81 and 89 BBCH.

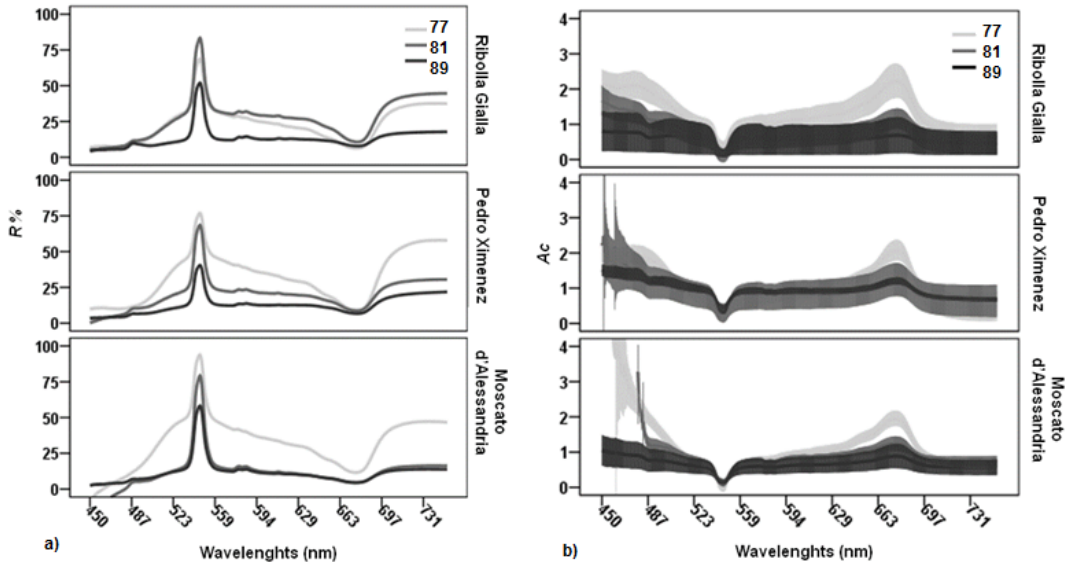


*Figure 13 a) Reflectance spectra (R%) for each accession during the three different phenological stages, with confidence interval at 95 %. Pre-veraison (77), Veraison (81), Harvest (89). B) Absorbance corrected in relation to the measured chlorophyll a (Ac) during the three different phenological stages for each accession, with relative errors.*

In this case the log (1/R) of each berry was rescaled according to the detected concentration of chlorophyll *a* (fig. 13b), as follows:

$Ac = (C_a)*(A_x)/A_{678}$

*Ac*: absorbance corrected in relation to the measured chlorophyll *a*; $C_a$: chlorophyll *a* concentration; $A_x$: absorbance at each wavelength; $A_{678}$: absorbance at 678 nm.

Since all photosynthetic pigments underwent a degradation during the ripening, in order to better understand their role in the color change, a comparison between the relative contribution of blue and red absorption bands, was necessary. With this objective the absorbance spectra were normalized by the chlorophyll *a* pick at maximum pick of absorbance 678nm.

$Ar = Ac_x/Ac_{678}$

Where *Ar* is the relative absorbance; $Ac_x$ is the *Ac* value at each wavelength; $Ac_{678}$ is the *Ac* value at 678 nm. Figure 14 shows a detail of the *Ar* (with standard error) over pre-veraison, veraison and harvest, for each accession.

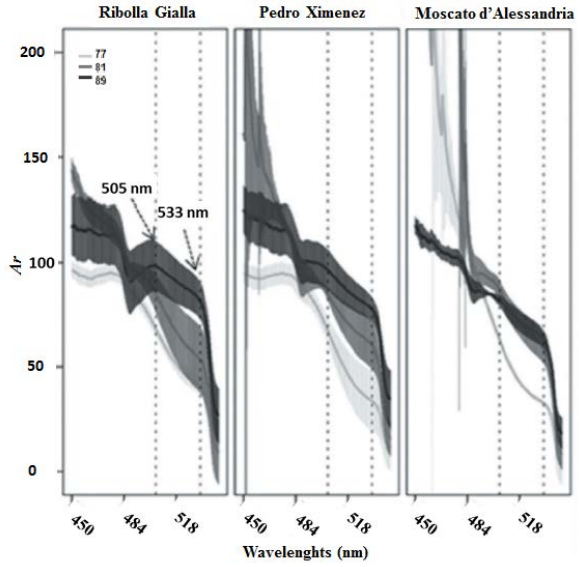


*Figure 14 Detail of the absorbance spectra proportional to the estimated chlorophyll a and normalized by its absorption maximum pick at 678 nm, during the phenological stages 77, 81 and 89 BBCH for each accession. Two maximum absorption picks are evidenced for the three accessions at 505and at 533 nm by dashed lines.*

Pigment degradation during ripening was confirmed by wet chemistry analysis (fig. 15). The bars represent, respectively, chlorophyll *a* (µg/cm$^2$), chlorophyll *b* (µg/cm$^2$), total chlorophylls (µg/cm$^2$) and carotenoids plus xanthophylls (µg/cm$^2$).

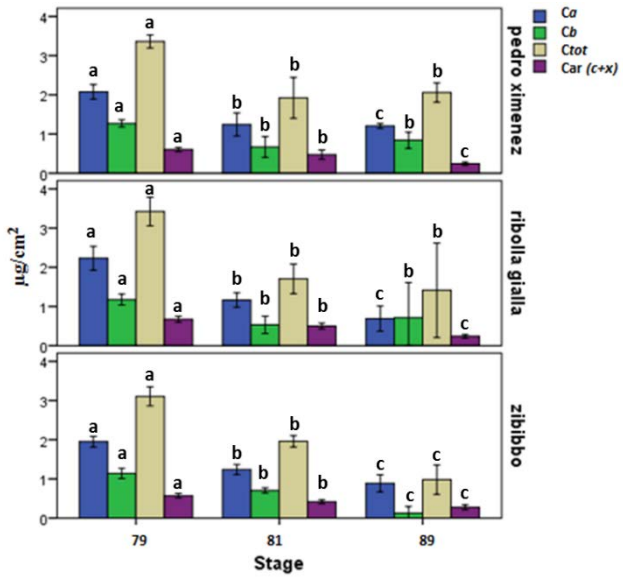


*Figure 15 Pigment content variation for the varieties Pedro Ximenez, Ribolla Gialla, Zibibbo, during the three different phenological stages (77, 81, 89 BBCH), with standard errors. The bars represent respectively chlorophyll a (µg/cm²), chlorophyll b (µg/cm²), total chlorophyll (µg/cm²) and carotenoids plus xanthophylls (µg/cm²). The letters represent the significance referred to the single compound.*

From pre-veraison to harvest, a decrease in all pigment concentration was visible for each accession.

On Chardonnay and Riesling, also, the sunburn susceptibility was tested, using the browning intensity index BI (Rustioni, *et al.* 2014) (fig. 16). The experiment was performed during the three phenological stages 77, 81 and 89 BBCH. However, only the first two stages are considered here since, because of the complete defoliation, all clusters were totally sunburned at harvest; thus, it was not possible to expose them at two different timings.

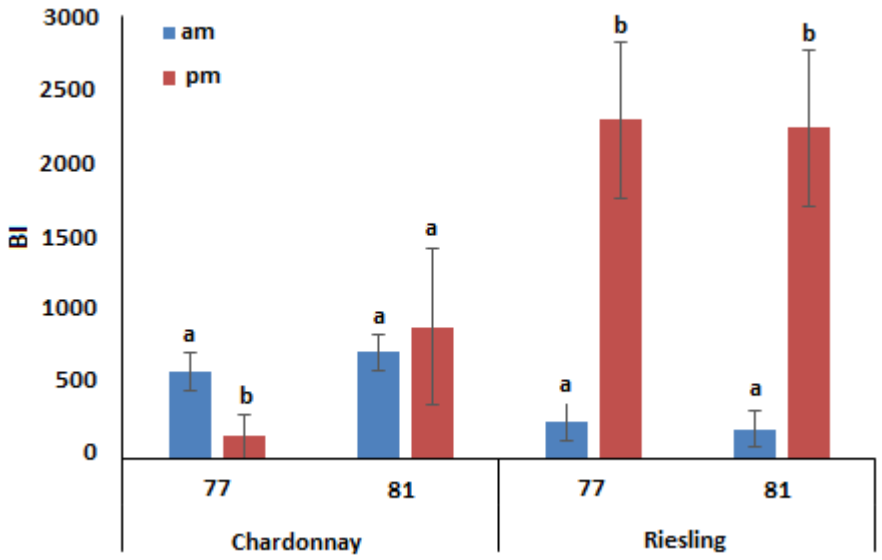


*Figure 16 Chardonnay and Riesling Browning Intensity Index, at 77 BBCH and 81 BBCH. The different color of the bars (± standard errors) represent the two exposure timing (am and pm). Letters are significant within phenological stage.*

In Chardonnay at 77 BBCH the BI was 591 and 145, respectively, for the am and the pm exposures; while at 81 it was 718 and 892. In Riesling at 77 BBCH the BI was 251 and 2298, respectively, for the am and the pm exposures; while at 81 it was 198 and 2245. Considering the correlation between chlorophyll index (*Chl*) (Merzlyak, *et al.* 2003b) and BI, proposed by Rustioni, *et al.* (2014), the chlorophyll was estimated on the control reflectance spectra (clusters not directly exposed to sunlight) used for the other experiment. It was 8.13 for Chardonnay at pre-veraison and 4.34 at veraison. For Riesling it was 8.44 and 4.35, respectively, at 77 and 81 BBCH.

## 2.3 Discussion

The general decrease in photosynthetic pigments during ripening is in agreement with other authors' findings (Baumes, *et al.* 2002).

The conversion of the reflectance spectra into their reciprocal was done considering that 1/R at certain wavelengths relates to pigment concentrations (Solovchenko, *et al.* 2010). The normalization at 800 nm was chosen, in agreement with literature (Merzlyak, *et al.* 2003b, Gitelson, *et al.* 2003), because plant pigments do not absorb at this wavelength.

To clarify the spectrum interpretation, figure 6 shows the theoretical absorbance contributions of chlorophyll *a*, *b* and their sum (*total chlorophylls*), which are drawn in the figure as dashed, dotted and solid grey lines respectively. The chromophore concentration should be proportional to the area under the Gaussian curve. Gaussian's area is proportional to the semi width, in correspondence to half of its height. However, the reflectance bandwidth was quite similar among our samples. Thus, the Gaussian's half height gleaned from the baseline, was used as main indicator of the light absorption intensity. The $(R_{800}/R_{675})$-$(R_{800}/R_{660})$ for chlorophyll *a* and $(R_{800}/R_{650})$-$(R_{800}/R_{630})$ for chlorophyll *b* were transformed into a logarithmic function because, in the visible spectral range, the relation between reflectance and leaf chlorophyll content is non-lineal (Buschmann and Nagel, 1993, Gitelson and Merzlyak, 1994). Whereas, the logarithm of the reciprocal reflectance was proved to be a good predictor for chlorophyll estimation in fresh leaves (Yoder and Pettigrew-Crosby, 1995). The regression between $C_aI$ and chlorophyll *a* ($\mu g/cm^2$) was higher than the one among $C_bI$ and chlorophyll *b* ($\mu g/cm^2$). This could be because of the lower concentration of this accessory pigment (Lichtenthaler, 1987). In general, chlorophyll *a* in respect to total chlorophylls varied from 63 to 67% during ripening. The indices proposed from other authors showed poor correlations with pigment contents. It is worth noting that they were developed on apple fruits and on leaves. In grapes, a general shift in pigment absorption bands, as well as possible interferences by different compounds, could explain the lack of correlation.

Saturation at certain wavelengths (e.g. in the carotenoids absorption region) should also be considered.

Data dispersion in figures 7, 8 and 9 could be related to the effect of pigment concentration variability within the individual skin portions, or to the biological variability of the berry itself. Another dispersion cause could be attributed to the surface estimation: grape berries are not perfect spheroids and a surface error might be expected.

The probable cause for the absence of correlation within $R_{800}/R_{530}$ index and carotenoids could be related to the absorption region of these compounds. Indeed, it overlaps that of many other grape pigments. Moreover, carotenoid absorption bands are closer to the lamp emission tail of the selected spectrophotometer; thus, reflectance measurements were less precise in this region. For these reasons it was not possible to identify direct relationships between reflectance spectra and carotenoid concentrations. The index was developed on the basis of the probable link between chlorophyll and carotenoid concentrations, due to their photosynthetic role, as well as to their common cell localization.

In figure 13*a* a general diminution in the absorption spectra is visible from pre-veraison to harvest. The absorption variation in the blue (450 - 490 nm) and in the red (630 -700 nm) during the ripening (fig. 13b), was in accordance with the measured degradation of photosynthetic pigments, shown in fig. 15. In the blue and in the red ranges, the absorbance differentiated between the first and the third stages (fig. 13b).

In fig. 14 the detail of the relative contribution of blue and red absorption bands not only confirmed the results obtained by wet chemistry (fig. 15), but also showed a different degradation intensity of the photosynthetic pigments. The rate of carotenoids in relation to the total pigments studied was 16% at pre-veraison, 20% at veraison and 31% at harvest. Similar results were obtained by other authors (Merzlyak, *et al*. 1998): in their cases a decrease in absorbance around 678nm was accompanied by a slight decrease in the blue range, characteristic of chlorophyll breakdown and carotenoid retention during leaf senescence and fruit ripening. At 450 - 500 nm (blue) in two of the three described

cases, the relative absorbance increased during ripening. From 500 to 550 nm a general increase in relative absorbance was visible during maturation. Two maximum, relative absorption picks appeared for the three accessions at 505 and 533 nm respectively. A spectral contribution at these wavelengths could be ascribed to oxidation products (Rustioni, *et al.* 2014), which also could play a significant role in white berry color modification during ripening.

The sunburn susceptibility for Chardonnay and Riesling are shown in figure 16. According to Rustioni, *et al.* (2014) the correlation between the chlorophyll index and browning index is nonlinear (it was described by a quadratic model). In pre-veraison, considering the estimated chlorophyll level, the Chardonnay was more affected by sunburn when it was exposed at 12.30 pm, while at veraison there was no difference. Riesling was more susceptible if exposed in the afternoon.

# Chapter 3 American experimentation

## 3.1 Materials and methods

### *3.1.1 Plant material and growth conditions*

The experiment was conducted during the 2014 growing season in two commercial vineyards located near Paterson, Columbia Valley, Washington, USA (lat. 45°57'N; long. 119°36'W; elevation 152 m). According to Fischer and Turner (1978) the Columbia Valley in Washington State may be classified as arid temperate steppe, with warm, very dry, and short summers and cold winters. Maximum summer temperatures may exceed 40°C. In winter the temperatures may drop below -20°C. The frost-free period lasts about 160 days. Usually the ripening season is characterized by warm to hot days and cool nights (daily range about 18°C). The annual precipitation (about 200 mm) is prohibitive for grape-growing without irrigation (Keller, *et al.* 2008).

Own-rooted *V. vinifera* cv. Chardonnay was planted in 1990, trained to a modified vertical shoot positioning system with bilateral cordon (0.8 m in height), and spur-pruned (3 buds per spur, 16 spurs per vine). The row-by-vine spacing was 3 m by 2 m. Own-rooted *V. vinifera* cv. Riesling was planted in 2003, trained to a modified vertical shoot positioning system with unilateral cordon (0.8 m in height), and spur-pruned (3 buds per spur, and 12 spurs per vine). The row by vine spacing was 3 m by 1.6 m. The row orientation in both vineyards was north-south. The vines were drip-irrigated, using two pressure-compensated emitters per vine with a flow rate of 2 L $h^{-1}$. Both vineyard blocks are relatively flat with loamy sand soil. Except for irrigation, other vineyard management practices were conducted based on the company's normal procedure. All vines were fully irrigated before budbreak. During the growing season, the amount of irrigation water was determined and applied (when necessary) weekly. Meteorological data and reference evapotranspiration ($ET_0$) were obtained from a Washington State University AgWeatherNet weather station located <5 km to the southwest of the experimental blocks (lat. 45°94'N; long. 119°66'W).

### *3.1.2 Experimental design and sample collection*

The experimental design consisted of a randomized block with two irrigation treatments with four replicates each. Full irrigation (FI): vines were irrigated to replace 100% crop evapotranspiration ($ET_c = K_c \times ET_0$, where $K_c$ was calculated according to Evans *et al.* 1993) from fruit set to harvest, with no water stress imposed. Deficit irrigation (DI): vines were irrigated to maintain a moderate water stress (stem water potential ($\Psi_{stem}$) at midday between -0.7 and -1 MPa) from fruit set to harvest. The $\Psi_{stem}$ was measured weekly using a pressure chamber (PMS Instrument Co., Albany, OR, USA). After harvest, soil moisture was replenished in both treatments.

For compositional analysis two cluster exposures were considered: one exposed to direct sunlight (sun), and the other totally shaded from sunlight (shade). The sun exposed clusters were chosen in the East side of the canopy, since on June 23, 2014 both blocks had hand leaf removal by the grower, exposing the overall bunch zone to direct sunlight. The shaded clusters were naturally covered by leaves, they were picked from the west side of the canopy. It was proved that one layer of *V. vinifera* leaves absorb 80 to 90% of incident solar radiation (Smart 1985), allowing predominantly diffuse light to strike the shaded clusters (Spayd *et al*., 2002).

Six temperature/light sensors (Hobo® Onset, Cape Cod, Massachusetts, USA) per irrigation treatment were randomly installed on 3 sun and 3 shade bunches. To prevent dislocation, each top and bottom part of the sensor was fixed on the cluster with black Scotch tape (to minimize the albedo of the tape material); the actual sensor zone was kept free from the tape. Sensors were installed before harvest (August 5$^{th}$ -August 20$^{th}$ and August 31$^{st}$ - September 15$^{th}$ for Chardonnay and Riesling, respectively). Minimum and maximum temperature ($T_{min}$ and $T_{max}$), and light intensity ($L_{max}$ and $L_{min}$) were recorded every 15 minutes. The number of hours with temperatures higher than 40°C (T > 40°C) and higher than 50°C (T > 50°C) were calculated.

Berry samples for skin compositional analysis (flavonols and flavanols) and soluble-solids determinations were collected during three phenological stages: pre-veraison (79 BBCH "majority of berries touching"), veraison (81 BBCH "beginning of ripening: berries begin to soft") and harvest (89 BBCH "berries ripe for harvest") (Lorenz, *et al*. 1995). Each sample consisted of 50 berries randomly sampled from four sun and four shaded clusters per irrigation treatment. The pedicel was left attached to prevent juice loss during transportation. In order to estimate the correlation between berry weight and diameter, additional 50 berries per replicate were sampled randomly. All berries were stored in sealable plastic bags and kept cooled. Once arrived at the laboratory the additional 50 berries were weighed and their diameters were measured. The other samples were stored at -80°C until the day of analysis.

### *3.1.3 Flavonol analysis*

The flavonols were extracted from the skins of both varieties. 20 berries were randomly sampled within the original 50 berry samples. They were weighed, and 4 skin disks (diameter 4 mm) were sampled from each berry (80 disks per sample) using a cork borer and immediately frozen under liquid nitrogen. The material was then stored at -80°C until analysed. Before extraction the skin disks were ground to a fine powder, using an automatic grinder (Mini BeadBeated-8, BioSpec Products; Bartlesville, OK, USA). The material was extracted in 5 mL of 50% methanol in water (v/v) for 20 minutes under sonication (modified from Downey and Rochfort 2008). The extracts were centrifuged (15 minutes at 10,000 rpm, 10°C). The supernatant was transferred to a microfuge tube and centrifuged for 5 minutes at 15,000 rpm. The final supernatant was then transferred to the HPLC auto-sampler vials.

The method was the one proposed by Downey *et al.* 2008 with some modifications: the gradients of the binary solvents used was set up in order to obtain a better separation in white skin grapes. An Agilent 1100 series HPLC-DAD system (Agilent Technologies, Palo Alto, CA, USA) was used for all chromatographic separations. The column temperature was maintained at 40°C. Separations were achieved using a binary gradient

of 10% formic acid in water (mobile phase A) and a 10% formic acid in methanol (mobile phase B). The column selected was a C-18 Zorbax RP 300 SB (250 mm x 4.6 mm, 5µm particle size; Agilent Technologies, Palo Alto, CA, USA) protected by a guard column of the same packing material. The flow rate was set at 1.0 mL/min. The gradient conditions were: 0-10 min, 100% A; 10-15 min, 10% B; 15-20 min, 20% B; 20-30 min, 30% B; 30-33 min, 40% B; 33-40 min, 100% B; followed by a 5-minute post-time run equilibration. Injection volume was 25 µL. The eluate was monitored by DAD at 280 and 353 nm. Manual peak identification was based on the retention time. Quantification was carried out using peak areas from external calibration curves. Where no standards were available, substances were quantified based on the retention time proposed in literature. The three standards used were quercetin 3-glucoside, quercetin 3-glucuronide and kaempferol 3-glucoside, all of which were provided by Sigma Aldrich.

### *3.1.4 Flavan-3-ol and proanthocyanidin analysis*

As for the flavonols, the flavanols were extracted from the skins of both cultivars. 20 berries were randomly sampled within the original 50 berry samples. They were weighed, and 4 skin disks (diameter 4 mm) were sampled from each berry (80 disks per sample), using a cork borer, quickly frozen in liquid nitrogen and then stored at -80°C until the analysis.

Each sample was extracted in 5 mL of 70% acetonitrile solution (99.99% Acetonitrile in 0.01% formic acid) and 30% of methanol solution (95% methanol in 4.99% ultrapure water and 0.01% formic acid) and then shaken for one hour (200 rpm). The extracts were then centrifuged (15 minutes at 10,000 rpm, 10°C). The supernatant was transferred to a microfuge tube and centrifuged for 5 minutes at 15,000 rpm. The final supernatant was then transferred to the HPLC auto-sampler vials.

To attain the better separation, different extraction methods, solvents and columns were tried. The best resolution and peak shape were achieved following the method proposed by Kelm, *et al*. 2006 on cacao and modified by Harbertson *et al.* 2014. The gradients of the solvent A and B were set up in order to obtain a better separation. An Agilent 1100

series HPLC-DAD system (Agilent Technologies, Palo Alto, CA, USA) was used for all chromatographic separations. The column temperature was maintained at 30°C. Separations were achieved using a binary gradient of 99.99% Acetonitrile in 0.01% Formic acid (mobile phase A) and a 95% Methanol in 4.99% distillate water and 0.01% Formic acid (mobile phase B). The column selected was a hydrophilic interaction liquid chromatography Luna HILIC (250 x 4.6 mm, surface area 200 sq. m/g; pore size 200 A; particle size 5µm; pH range 2.0-7.5; Phenomenex) protected by a guard column of the same packing material. The flow rate was set at 0.8 mL/min. The gradient conditions for separation were: 0-35 min, 100% A; 35-40 min, 45% B; 40-45 min, 45% B; 45 min, 100% A; followed by a 5-minute post-time run equilibration. Injection volume was 25 µL. Elute was monitored by DAD at 280 and 353 nm. Manual peak identification was based on the retention time. Quantification was carried out using peak areas from external calibration curves. The catechin standard used was purchased from Sigma Aldrich.

The different-sized-flavanols were recognized according to their retention time (Kelm *et al.*, 2006). Total flavanols were expressed as sum of monomers, oligomers and polymers.

### *3.1.5 Tannin analysis*

This method uses bovine-serum albumin precipitation to allow measurement of tannins using a spectrophotometer. It was adapted by Harbertson *et al.* (2002), from the original method for the quantitative determination of tannins proposed by Hagerman and Butler 1978.

For skin extraction 10 berries were collected randomly from each sample and weighed. Each berry was sliced transversely around the equator (Harbertson *et al.*, 2002) without cutting the mesocarp. To reduce the presence of the pulp, the skins were gently pooled on tissue paper and after 10 hours put into a 50 mL Eppendorf tube and frozen at -80°C until the extraction. For the extraction 10 mL of a 70% acetone in water (v/v) were added to the skin samples and shaken gently (100 rpm) overnight. The next morning the samples were centrifuged for 5 min (10,000 rpm), the supernatant solution was collected in previously-weighed polyvac clear vials and the acetone was removed at 38°C using a

vacuum polyvac at 500 bar. The remaining aqueous extract containing the tannins was weighed (vials included) and transferred into plastic vials and frozen at -80 °C until the analysis.

This method uses different buffers and solutions. The reagents used for their preparation were: bovine-serum albumin (BSA, fraction V power), sodium dodecyl sulphate (SDS; lauryl sulphate, sodium salt), triethanolamine (TEA), ferric chloride hexahydrate ($FeCl_3*6H_2O$), sodium chloride (NaCl) and (+) - catechin, purchased from Sigma-Aldrich (St. Louis, MO). The buffers are composed as listed below.

Buffer A: 200 mM of glacial acetic acid and 170 nM NaCl, and pH was adjusted to 4.9 with sodium hydroxide (NaOH).

Buffer B: 5 g/L potassium bitartrate (KHTa), 12 % ethanol and pH was adjusted to 3.3 with HCl.

Buffer C: 5% TEA (v/v), 5 % SDS (w/v) and pH was adjusted to 9.4 with chloridric acid (HCl).

Protein solution: 1 mg/mL BSA dissolved in buffer A and stored at 4°C.

Ferric Chloride reagent: 0.01 N HCl, 10 mM $FeCl_3$.

Catechin standard: 1 mg/mL (+) - catechin solution dissolved in a 10 % ethanol.

The incubation was performed at room temperature. To prepare the standard curve (+)-catechin samples, in the range of 50 µL to 300 µL of standard catechin solution, were taken and the volume was adjusted to 875 µL with Buffer C into a 2 mL microfuge tube. Then, 125 µL of the ferric chloride reagent were added. A zero catechin sample was made by adding 125 µL of the $FeCl_3$ reagent to 875 µL of buffer C and the absorbance was read at 510 nm using an Agilent diode array spectrophotometer (Model Number 8453; Agilent Technologies, Santa Clara, CA). This absorbance was then used to be subtracted from the

absorbance obtained from the standard samples and the skin samples. Everything must be incubated for 10 minutes before reading them out.

The variable amount of skin extract was diluted in Buffer B (500 µL in total in a microfuge tube), depending on the phenological stage and the varieties, this in order to give tannins values in the range of the standard curve. For each diluted sample, 1,000 µL of BSA (protein solution) were added; everything was incubated for 15 minutes, then centrifuged for 5 minutes at 14,000 rpm. The supernatant was carefully poured off in order to retain the pellet in the microfuge tube. 875 µL of the Buffer C were added to the pelleted sample and incubated for 10 minutes. Each tube was vortexed until the complete dissolution of the pellet and let stand for 10 minutes. Then the absorbance at 510 nm was read (background reading) in an Agilent diode array spectrophotometer (Model Number 8453; Agilent Technologies, Santa Clara, CA). After recording the value, 125 µL of the ferric chloride reagent were added, mixing carefully with all the sample incubated for 10 minutes, and the absorbance at 510 nm was reread. The concentration of tannins in skin samples was calculated using the standard curve, after the background absorbance was subtracted from the final absorbance. The general equation to determine the concentration of tannin was:

$2\left(\frac{Abs-intercept}{slope}\right)(dilution)$= mg/L CE (Catechin Equivalent)

### *3.1.6 Statistical analysis*

Results were evaluated using a multivariate analysis of variance (ANOVA), the considered factors were the stages (79, 81, 89 BBCH), the exposures (shade and sun) and the irrigations (DI and FI). When the interactions between the dependent variable were significant, a one way ANOVA was used to analyze the contribution of each factors. Post-hoc pair-wise comparisons were performed by a Turkey-b's test, only for factors with more than two levels. Statistical testing was performed with SPSS© (PASW Statistics 21 version, SPSS Inc. Chicago, Illinois).

## 3.2 Results

The Chardonnay total soluble solids (%) and berry weight (g), increased from pre-veraison to harvest ($P<0.05$) (tab. 2). The exposure and irrigation treatments, did not affect the sugar level. Berry weight was higher in FI treatment. In Chardonnay 79, 81 and 89 BBCH were respectively July 29, August 7 and September 9.

*Table 2 Chardonnay soluble solids (%) and berry weight (g), mean values ±standard errors during three phenological stages (79, 81, 89 BBCH), irrigation (DI and FI) and exposure (sun and shade) treatments.*

| | | | Phenology | | |
|---|---|---|---|---|---|
| **Variable** | **Exposure** | **Irrigation** | **79** | **81** | **89** |
| **Soluble solids (%)** | **shade** | **DI** | 5.8 ±0.1 | 12.3 ±1.2 | 22.2 ±1.2 |
| | | **FI** | 5.2 ±0.3 | 12.2 ±0.8 | 21.9 ±0.4 |
| | **sun** | **DI** | 6.3 ±0.5 | 14.5 ±0.6 | 22.8 ±0.2 |
| | | **FI** | 5.3 ±0.2 | 14.7 ±1 | 22.1 ±0.8 |
| **Berry (g)** | **shade** | **DI** | 0.71 ±0.4 | 1.1 ±0.3 | 1.3 ±0.1 |
| | | **FI** | 0.94 ±0.3 | 1.3 ±0.1 | 1.5 ±0.4 |
| | **sun** | **DI** | 0.69 ±0.2 | 1.1 ±0.4 | 1.2 ±0.2 |
| | | **FI** | 0.87 ±0.4 | 1.2 ±0.2 | 1.4 ±0.1 |

The Riesling total soluble solids (%) and berry weight (g), increased from pre-veraison to harvest ($P<0.05$) (tab. 3). The exposure and irrigation treatments, did not affect the sugar level and the berry weight. In Riesling 79, 81 and 89 BBCH were respectively August 15, August 19, and September 23.

*Table 3 Riesling: soluble solids (%) and berry weight (g), mean values ±standard errors during three phenological stages (79, 81, 89 BBCH), irrigation (DI and FI) and exposure (sun and shade) treatments.*

| | | | Phenology | | |
|---|---|---|---|---|---|
| **Variable** | **Exposure** | **Irrigation** | **79** | **81** | **89** |
| **Soluble solids (%)** | **shade** | **DI** | 5.4 ±0.4 | 14.4 ±0.3 | 21.1 ±1.7 |
| | | **FI** | 5.0 ±0.4 | 11.9 ±1.2 | 19.8 ±1.2 |
| | **sun** | **DI** | 5.5 ±0.2 | 15.1 ±0.4 | 20.2 ±0.8 |
| | | **FI** | 5.2 ±0.3 | 12.7 ±0.9 | 19.8 ±0.4 |
| **Berry (g)** | **shade** | **DI** | 0.58 ±0.1 | 1.1 ±1.2 | 1.4 ±1.2 |
| | | **FI** | 0.64 ±0.3 | 1.1 ±0.8 | 1.4±0.4 |
| | **sun** | **DI** | 0.76 ±0.5 | 1.1 ±0.6 | 1.3 ±0.2 |
| | | **FI** | 0.86 ±0.2 | 1.1 ±1.1 | 1.5 ±0.8 |

In terms of absolute concentration of phenolics, several statistical tests indicated significant effects from exposure and phenological stages and their interaction in both Chardonnay (tab. 4 and 5) and Riesling (tab. 6 and 7). The HPLC results are expressed in concentration, since the contribution to the variance of phenology, exposure and irrigation, were similar when expressed on a per berry basis. This was not the case for skin tannins measured by spectrophotometer.

*Table 4 Chardonnay: multiway analysis of variance in flavonols analysed by HPLC, considering the phenology, the exposure and irrigation treatments. The values are significant for P<0.05*

| | Total Flavonols (µg/cm$^2$ CE) | Quercetin glucoside (µg/cm$^2$ CE) | Quercetin glucuronide (µg/cm$^2$ CE) | Kaempferol-types (µg/cm2) |
|---|---|---|---|---|
| Phenology | 0.00 | 0.00 | 0.85 | 0.00 |
| Exposure | 0.00 | 0.00 | 0.00 | 0.00 |
| Irrigation | 0.01 | 0.07 | 0.53 | 0.42 |
| Phenology * Exposure | 0.00 | 0.00 | 0.73 | 0.00 |
| Phenology * Irrigation | 0.00 | 0.04 | 0.18 | 0.97 |
| Exposure*Irrigation | 0.32 | 0.12 | 0.11 | 0.29 |
| Phenology * Exposure * Irrigation | 0.05 | 0.11 | 0.61 | 0.88 |
| Error SS | 1366691 | 2714802 | 151474 | 810196 |
| Error MS | 37964 | 75411 | 4208 | 22505 |

*Table 5 Chardonnay: multiway analysis of variance in flavanols, considering the phenology, the exposure and irrigation treatments. Monomers, dimers, trimers and polymers were analysed by HPLC; while skin tannin expressed as mg/berry and mg/g FW refers to the spectophotometer analysis. The values are significant for P<0.05*

| | Monomers (µg/cm$^2$ CE) | Dimers (µg/cm$^2$ CE) | Trimers (µg/cm$^2$ CE) | Polymers (µg/cm$^2$ CE) | Skin Tannin mg/berry | Skin Tannin mg/g FW |
|---|---|---|---|---|---|---|
| Phenology | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Exposure | 0.62 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Irrigation | 0.46 | 0.88 | 0.14 | 0.26 | 0.76 | 0.00 |
| Phenology * Exposure | 0.22 | 0.00 | 0.01 | 0.04 | 0.00 | 0.00 |
| Phenology * Irrigation | 0.44 | 0.14 | 0.52 | 0.44 | 0.10 | 0.00 |
| Exposure*Irrigation | 0.48 | 0.94 | 0.93 | 0.76 | 0.29 | 0.07 |
| Phenology * Exposure * Irrigation | 0.34 | 0.63 | 0.34 | 0.60 | 0.24 | 0.17 |
| Error SS | 11983127 | 897505 | 170926 | 3458944 | 0.12 | 0.10 |
| Error MS | 332865 | 24931 | 4748 | 96082 | 0.00 | 0.00 |

*Table 6 Riesling: multiway analysis of variance in flavonols analysed by HPLC, considering the phenology, the exposure and irrigation treatments. The values are significant for P<0.05*

| | Total Flavonols (µg/cm$^2$ CE) | Quercetin glucoside (µg/cm$^2$ CE) | Quercetin glucuronide (µg/cm$^2$ CE) | Kaempferol-types (µg/cm2) |
|---|---|---|---|---|
| Phenology | 0.00 | 0.00 | 0.03 | 0.00 |
| Exposure | 0.00 | 0.00 | 0.00 | 0.00 |
| Irrigation | 0.91 | 0.88 | 0.91 | 0.93 |
| Phenology * Exposure | 0.00 | 0.00 | 0.60 | 0.00 |
| Phenology * Irrigation | 0.77 | 0.94 | 0.35 | 0.76 |
| Exposure*Irrigation | 0.69 | 0.35 | 0.33 | 0.90 |
| Phenology * Exposure * Irrigation | 0.73 | 0.62 | 0.47 | 0.87 |
| Error SS | 6533021 | 617993 | 333924 | 1055930 |
| Error MS | 181473 | 17166 | 9276 | 29331 |

*Table 7 Riesling: multiway analysis of variance in flavanols, considering the phenology, the exposure and irrigation treatments. Monomers, dimers, trimers and polymers were*

*analysed by HPLC; while skin tannin expressed as mg/berry and mg/g FW refers to the spectophotometer analysis. The values are significant for P<0.05*

| | Monomers (µg/cm$^2$ CE) | Dimers (µg/cm$^2$ CE) | Trimers (µg/cm$^2$ CE) | Polymers (µg/cm$^2$ CE) | Skin Tannin mg/berry | Skin Tannin mg/g FW |
|---|---|---|---|---|---|---|
| Phenology | 0.00 | 0.69 | 0.00 | 0.00 | 0.00 | 0.53 |
| Exposure | 0.00 | 0.01 | 0.00 | 0.02 | 0.01 | 0.13 |
| Irrigation | 0.67 | 0.52 | 0.78 | 0.14 | 0.00 | 0.01 |
| Phenology * Exposure | 0.00 | 0.03 | 0.03 | 0.02 | 0.04 | 0.04 |
| Phenology * Irrigation | 0.83 | 0.47 | 0.45 | 0.02 | 0.00 | 0.00 |
| Exposure*Irrigation | 0.43 | 0.42 | 0.11 | 0.01 | 0.71 | 0.96 |
| Phenology * Exposure * Irrigation | 0.53 | 0.23 | 0.15 | 0.00 | 0.45 | 0.33 |
| Error SS | 72938 | 94718 | 7808 | 5416 | 0.09 | 0.09 |
| Error MS | 2026 | 2631 | 217 | 150 | 0.00 | 0.00 |

In Chardonnay the total flavonols increased significantly in the three phenological stages, especially in sun exposed berries (83.5%, 80.5% and 87.7% respectively at 79, 81 and 89 BBCH) as compared to shade berries (P<0.05) (table 8).

*Table 8 Chardonnay skin flavonols concentration (µg/cm$^2$) identified by HPLC and expressed as mean values ± standard errors, considering the exposure (sun and shade),*

*the irrigation (DI and FI) treatments and the phenological stages (79, 81, 89 BBCH); P<0.05. Total flavonols are expressed as quercetin glucoside equivalents (QGE).*

| Variable | Exposure | Irrigation | Phenology 79 | 81 | 89 |
|---|---|---|---|---|---|
| Total Flavonols (µg/cm$^2$ QGE) | shade | DI | 7.6 ±2.1 | 7.3 ±3.2 | 14.7 ±1.3 |
| | | FI | 5.6 ±1.2 | 9.4 ±2.2 | 9.7 ±5.2 |
| | sun | DI | 40.0 ±8.6 | 39.4 ±14 | 113.8 ±20.6 |
| | | FI | 39.8 ±10 | 46.3 ±8.8 | 85.2 ±8.4 |
| Quercetin glucoside (µg/cm$^2$) | shade | DI | 1.9 ±0.3 | 2.1 ±0.9 | 5.7 ±2.3 |
| | | FI | 1.1 ±0.8 | 2.6 ±0.6 | 2.8 ±0.5 |
| | sun | DI | 13.3 ±3.0 | 14.9 ±5.6 | 70.1 ±17.7 |
| | | FI | 12.6 ±3.3 | 16.7 ±3.7 | 38.5 ±3.6 |
| Quercetin glucuronide (µg/cm$^2$) | shade | DI | 3.4 ±1.3 | 2.6 ±1.2 | 5.2 ±2.1 |
| | | FI | 2.3 ±0.9 | 3.6 ±0.8 | 1.8 ±0.3 |
| | sun | DI | 14.7 ±3.1 | 11.4 ±3.7 | 12.6 ±5.3 |
| | | FI | 14.8 ±3.5 | 16.5 ±3.2 | 13.8 ±1.6 |
| Kaempferol-types (µg/cm$^2$) | shade | DI | 0.2 ±0.2 | 0.7 ±0.2 | 3.9 ±1.5 |
| | | FI | 0.5 ±1.0 | 0.4 ±0.2 | 5.5 ±8.6 |
| | sun | DI | 6 ±3.7 | 5.1 ±4.9 | 31.8 ±18.9 |
| | | FI | 1 ±1.2 | 3.1 ±1.4 | 26.2 ±12.1 |

Quercetin glucoside was about 92.1% higher in sun exposed berries at harvest (89 BBCH) as compared to shade berries (P<0.05) (table 8). The levels of quercetin glucuronide did not change over the season, with 77.3% higher mean concentration in sun exposed berries as compared to shade berries (P<0.05) (table 8). The analysis of variance indicated significant effects attributable to exposure and phenological stages (P<0.05) (fig. 17 a, b), but no effects were due to the irrigation regimes. Kaempferol-types increased about 83.8% higher in sun exposed berries at harvest (89 BBCH) as compared to shade berries (P<0.05) (table 8).

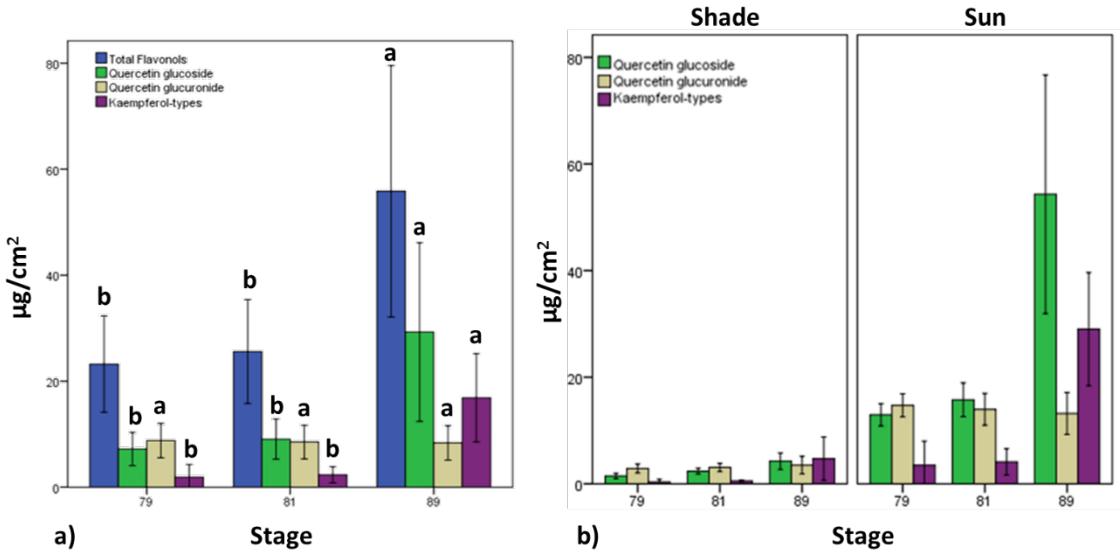


*Figure 17 Chardonnay: flavonol concentrations (µg/cm²) over the phenological stages(79, 81, 89 BBCH) (a) and considering the exposures (shade and sun) (b). The bars indicate the different flavonol compounds ±standard errors. Different letters within the bars indicate significant differences between the same compound during the three phenological stages at P<0.05.*

In figure 18 shows an example of flavanol chromatogram.

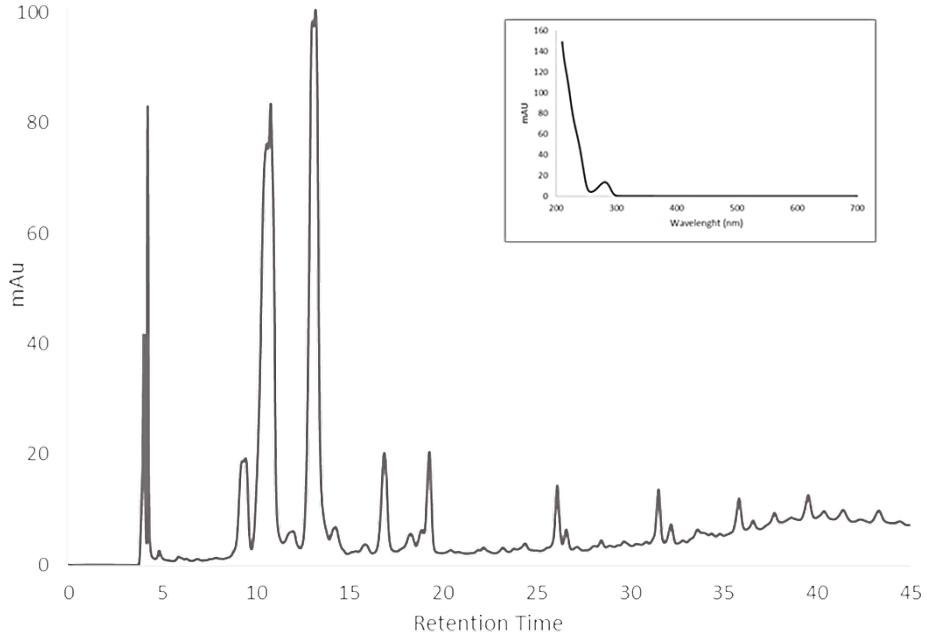


*Figure 18 Example of flavanol chromatogram. Monomers eluted within 15 minutes, dimers 15- 25 minutes, trimers 25-30 minutes and polymers after 30 minutes. The inset indicates the typical flavanol spectrum shape, with maximum absorbance peak at 280 nm.*

In Chardonnay total flavanols, monomers, dimers, trimers and polymers, decreased from pre-veraison to harvest (table 9).

*Table 9 Chardonnay skin flavanols, concentration µg/cm² (expressed as catechin equivalent CE) identified by HPLC and shown as mean values ± standard errors, considering the exposure (sun and shade), the irrigation (DI and FI) treatments and the*

*phenological stages (79, 81, 89 BBCH); P<0.05. Total flavanols are expressed as sum of monomers, dimers, trimers and polymers.*

| Variable | Exposure | Irrigation | Phenology 79 | 81 | 89 |
|---|---|---|---|---|---|
| Total Flavanols (µg/cm$^2$ CE) | shade | DI | 32.9 ±5.0 | 29.5 ±4.7 | 7.7 ±1.9 |
| | | FI | 28.8 ±5.4 | 23.5 ±10.5 | 7.7 ±1.7 |
| | sun | DI | 54.3 ±7.3 | 31.8 ±6.2 | 29.7 ±12.2 |
| | | FI | 48.9 ±7.5 | 27.1 ±3.5 | 32.1 ±11.6 |
| Monomers (µg/cm$^2$ CE) | shade | DI | 8.2 ±1.6 | 16.9 ±3.4 | 2.5 ±1.8 |
| | | FI | 9.6 ±5.0 | 10.7 ±5.7 | 2.3 ±2.2 |
| | sun | DI | 11.3 ±1.0 | 11.8 ±0.6 | 10.5 ±2.2 |
| | | FI | 11.0 ±2.6 | 13.7 ±1.4 | 11.1 ±0.4 |
| Dimers (µg/cm$^2$ CE) | shade | DI | 7.8 ±1.4 | 3.1 ±1.6 | 0.8 ±0.3 |
| | | FI | 5.9 ±0.7 | 3.5 ±1.0 | 1.9 ±2.5 |
| | sun | DI | 12.6 ±1.5 | 4.6 ±0.7 | 6.4 ±3.5 |
| | | FI | 11.5 ±0.9 | 3.3 ±1.1 | 8.6 ±3.8 |
| Trimers (µg/cm$^2$ CE) | shade | DI | 4.8 ±0.8 | 2.6 ±1.1 | 1.3 ±0.3 |
| | | FI | 3.8 ±1.0 | 2.7 ±0.4 | 0.9 ±0.2 |
| | sun | DI | 7.1 ±1.1 | 3.9 ±0.8 | 4.0 ±1.2 |
| | | FI | 6.6 ±1.3 | 2.8 ±0.6 | 4.4 ±1.8 |
| Polymers (µg/cm$^2$ CE) | shade | DI | 12.1 ±1.4 | 6.9 ±3.3 | 3.1 ±0.5 |
| | | FI | 9.5 ±2.5 | 6.7 ±1.1 | 2.7 ±0.6 |
| | sun | DI | 23.3 ±5.5 | 11.3 ±4.6 | 8.9 ±4.7 |
| | | FI | 19.8 ±5.1 | 7.2 ±1.3 | 10.9 ±7.6 |

In Chardonnay the total flavanols increased significantly at pre-veraison and harvest (79 and 89 BBCH, respectively), especially in sun exposed berries (40.1% and 75.1% respectively) as compared to shade berries (P<0.05) (table 9). Monomers were about 20.5% and 77.8% higher in sun exposed berries at pre-veraison and harvest as compared to shade berries (P<0.05) (table 9). The level of dimers were 43% higher at pre-veraison and 81.3% higher at harvest in sun exposed berries as compared to shade berries (P<0.05) (table 9). Trimers were about 37.7% and 73.8% higher in sun exposed berries at pre-veraison and harvest as compared to shade berries (P<0.05) (table 9). Polymers increased about 50% higher at pre-veraison and 70.7% higher at harvest in sun exposed berries as compared to shade berries (P<0.05) (table 9). As for flavonols, the analysis of variance

for the flavanols showed significant effect just for exposures and phenological stages (P<0.05) (fig. 19).

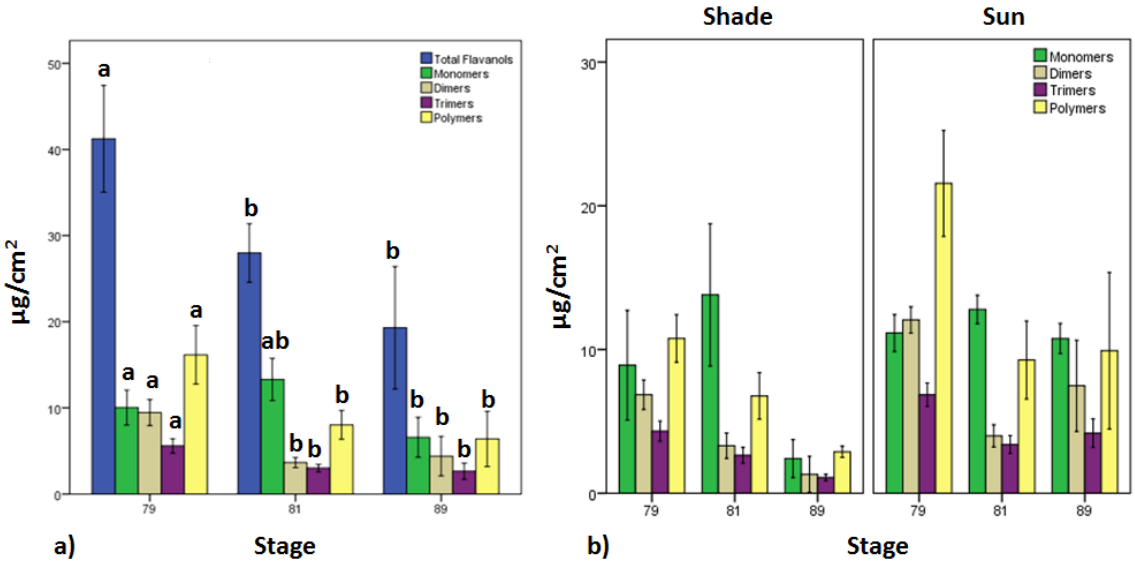


*Figure 19 Chardonnay flavanol concentrations (µg/cm²) expressed as catechin equivalent (CE) over the phenological stages (79, 81, 89 BBCH) (a) and considering the exposures (shade and sun) (b). The bars indicate the different flavanol compounds ±standard errors. Total flavanols are expressed as sum of monomers, dimers, trimers and polymers.*

Chardonnay skin tannins (mg/berry and mg/g FW) analysed by spectrophotometer showed a 37.2% decrease over the growing season from 79 to 89 BBCH in shade DI (P<0.05) (table 10). In FI the levels were constant from pre-veraison to harvest. In general, sun exposed berries had higher (P<0.05) concentrations, with decreasing patterns starting after veraison. The irrigation treatments were only significant on a per mg/g FW basis, with higher concentrations in DI, especially in the sun and decreasing 26.9 % over the season (from 79 to 89 BBCH) (table 10 and fig. 20).

*Table 10 Chardonnay skin tannins, mean values and standard deviations (P<0.05) over the three phenologycal stages (79, 81, 89 BBCH), and considering the two exposure (shade and sun) and irrigation treatments (DI and FI). Data are considered as mg/berry and mg/g FW. The values are espressed as cathechin equivalents.*

| | | | Phenology | | |
|---|---|---|---|---|---|
| **Variable** | **Exposure** | **Irrigation** | **79** | **81** | **89** |
| **Skin Tannins (mg/ berry)** | **shade** | **DI** | 0.28 ±0.02 | 0.27 ±0.05 | 0.3 ±0.02 |
| | | **FI** | 0.18 ±0.02 | 0.3 ±0.03 | 0.33 ±0.03 |
| | **sun** | **DI** | 0.36 ±0.07 | 0.27 ±0.03 | 0.45 ±0.11 |
| | | **FI** | 0.38 ±0.05 | 0.35 ±0.08 | 0.46 ±0.07 |
| **Skin Tannins (mg/g FW)** | **shade** | **DI** | 0.43 ±0.04 | 0.29 ±0.06 | 0.27 ±0.03 |
| | | **FI** | 0.19 ±0.03 | 0.24 ±0.03 | 0.22 ±0.03 |
| | **sun** | **DI** | 0.52 ±0.09 | 0.26 ±0.02 | 0.38 ±0.08 |
| | | **FI** | 0.41 ±0.08 | 0.25 ±0.04 | 0.32 ±0.06 |

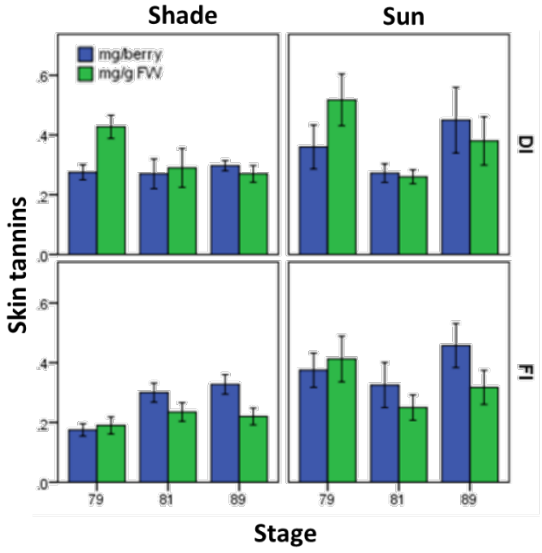


*Figure 20 Chardonnay: skin tannins showed as content and concentration ±standard errors during three phenological stages (79, 81, 89 BBCH), irrigation (DI and FI) and exposure (sun and shade) treatments.*

Looking at the data on a per berry basis, a slight increase was reported over the season.

Riesling skin flavonols are shown in table 11, the mean values and standard errors considering the three phenological stages, the exposure and irrigation treatments. Total flavonols are expressed as quercetin glucoside equivalents (QGE). They did not show significant changes over the season or between irrigation treatments ($P<0.05$).

*Table 11 Riesling skin flavonols concentration (µg/cm$^2$) identified by HPLC and expressed as mean values ± standard errors, considering the exposure (sun and shade), the irrigation (DI and FI) treatments and the phenological stages (79, 81, 89 BBCH); P<0.05. Total flavonols are expressed as quercetin glucoside equivalents (QGE).*

| | | | Phenology | | |
|---|---|---|---|---|---|
| **Variable** | **Exposure** | **Irrigation** | **79** | **81** | **89** |
| **Total Flavonols (µg/cm$^2$ QGE)** | **shade** | **DI** | 14.1 ±5.3 | 8.8 ±6.0 | 13.6 ±4.6 |
| | | **FI** | 23.9 ±17.1 | 6.9 ±2.5 | 13.9 ±7.5 |
| | **sun** | **DI** | 55 ±18.1 | 52.4 ±22.1 | 92.1 ±31.1 |
| | | **FI** | 52.1 ±6.2 | 51.3 ±21.0 | 84.4 ±32.2 |
| **Quercetin glucoside (µg/cm$^2$)** | **shade** | **DI** | 2.9 ±1.1 | 2.2 ±1.4 | 5.1 ±2.5 |
| | | **FI** | 6.3 ±6.1 | 2.7 ±3.1 | 4.8 ±3.0 |
| | **sun** | **DI** | 14.7 ±5.3 | 16.3 ±7.3 | 33.9 ±10.1 |
| | | **FI** | 10.3 ±6.9 | 15.3 ±8.9 | 33.2 ±12.1 |
| **Quercetin glucuronide (µg/cm$^2$)** | **shade** | **DI** | 6.7 ±2.4 | 3.6 ±2.2 | 3.4 ±1.1 |
| | | **FI** | 10.6 ±7.0 | 3.1 ±1.0 | 4.5 ±2.0 |
| | **sun** | **DI** | 21.1 ±6.3 | 17.2 ±6.7 | 23.4 ±4.4 |
| | | **FI** | 22.4 ±4.3 | 18.2 ±6.8 | 17.6 ±6.0 |
| **Kaempferol-types (µg/cm$^2$)** | **shade** | **DI** | 0.2 ±0.1 | 0.7 ±0.8 | 3.1 ±1.1 |
| | | **FI** | 2 ±0.3 | 0.1 ±0.2 | 3.9 ±3.6 |
| | **sun** | **DI** | 4.7 ±3.7 | 9.8 ±5.4 | 26.6 ±20.1 |
| | | **FI** | 3.3 ±2.9 | 5.8 ±4.3 | 30.3 ±18.1 |

In Riesling the total flavonols did not change over the season (from 79 to 89 BBCH), with a 77.9% higher mean concentration in sun exposed berries as compared to shade berries ($P<0.05$) (table 11). Quercetin glucoside increased significantly in the three phenological

stages, especially in sun exposed berries (63.2%, 84.2% and 85.1% respectively at 79, 81 and 89 BBCH) as compared to shade berries (P<0.05) (table 11). The levels of quercetin glucuronide did not change over the season, with a 73.4% higher mean concentration in sun exposed berries as compared to shade berries (P<0.05) (table 11). Kaempferol-types increased over the season, especially in sun exposed berries (72.5%, 94.9% and 87.7% respectively at 79, 81 and 89 BBCH) as compared to shade berries (P<0.05) (table 11).

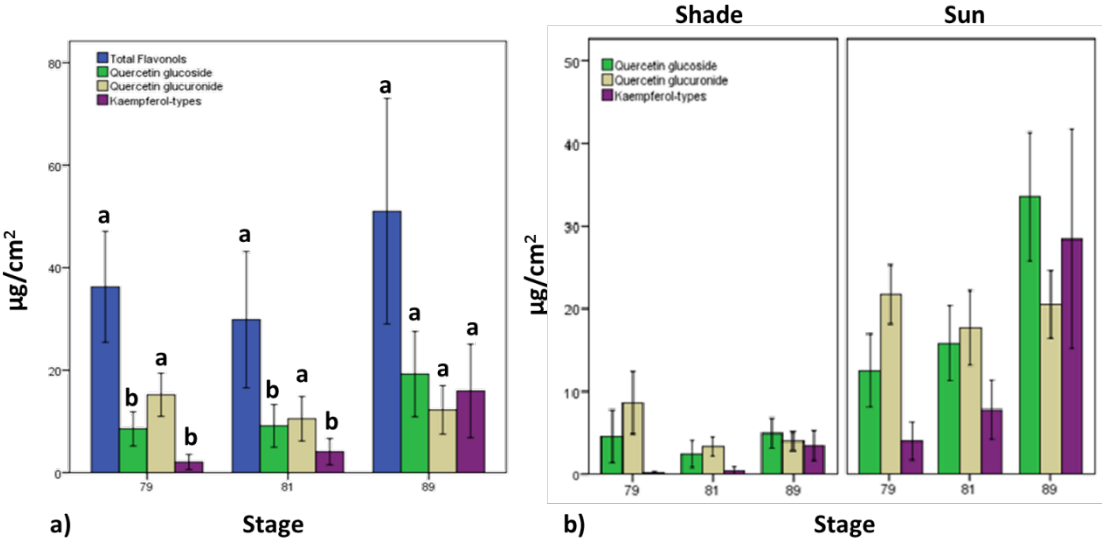


*Figure 21 Riesling: flavonol concentrations (µg/cm²) over the phenological stages (79, 81, 89 BBCH) (a) and considering the exposures (shade and sun) (b). The bars indicate the different flavonol compounds ±standard errors. Different letters within the bars indicate significant differences between the same compound during the three phenological stages at P<0.05.*

Total flavanols showed an increase in sun-exposed berries from pre-veraison to harvest in the range from 10 to 20 (µg/cm$^2$ CE) in DI and from 7 to 19 (µg/cm$^2$ CE) in FI (table 12). Monomers were not detectable at 79 and 81 BBCH in both exposure and irrigation treatments. Their concentration at harvest were 2 and 3 (µg/cm$^2$ CE) respectively for DI and FI in the shade, and 12 and 9 (µg/cm$^2$ CE) respectively for DI and FI in the sun.

*Table 12 Riesling skin flavanols, concentration µg/cm² (expressed as catechin equivalent CE) identified by HPLC and shown as mean values ± standard errors, considering the exposure (sun and shade), the irrigation (DI and FI) treatments and the phenological stages (79, 81, 89 BBCH); P<0.05. Total flavanols are expressed as sum of monomers, dimers, trimers and polymers.*

| | | | Phenology | | |
|---|---|---|---|---|---|
| **Variable** | **Exposure** | **Irrigation** | **79** | **81** | **89** |
| **Total Flavanols (µg/cm² CE)** | **shade** | **DI** | 6.6 ±1.8 | 5 ±3.5 | 8.1 ±5.7 |
| | | **FI** | 8.2 ±2.9 | 4.7 ±2.4 | 7.5 ±3.6 |
| | **sun** | **DI** | 9.8 ±0.9 | 5.6 ±3.7 | 19.5 ±6.5 |
| | | **FI** | 6.7 ±2.14 | 6.9 ±3.4 | 19.4 ±6.8 |
| **Monomers (µg/cm² CE)** | **shade** | **DI** | 0 ±0.0 | 0 ±0.0 | 2.3 ±4.5 |
| | | **FI** | 0 ± 0.0 | 0 ± 0.0 | 3.1 ±2.3 |
| | **sun** | **DI** | 0 ± 0.0 | 0 ± 0.0 | 11.5 ±2.1 |
| | | **FI** | 0 ± 0.0 | 0 ± 0.0 | 8.7 ±6.5 |
| **Dimers (µg/cm² CE)** | **shade** | **DI** | 4.1 ±1.6 | 4 ±2.9 | 2.6 ±0.6 |
| | | **FI** | 4.6 ±1.9 | 3.7 ±2.6 | 2 ±0.5 |
| | **sun** | **DI** | 5.5 ±1.7 | 4.1 ±2.4 | 5.5 ±3.6 |
| | | **FI** | 4.1 ±1.9 | 4.7 ±3.0 | 7.2 ±2.3 |
| **Trimers (µg/cm² CE)** | **shade** | **DI** | 1.5 ±0.1 | 0.6 ±0.4 | 0.2 ±0.3 |
| | | **FI** | 2.4 ±0.8 | 0.8 ±0.1 | 0 ±0.0 |
| | **sun** | **DI** | 3.1 ±0.7 | 1.2 ±0.9 | 2.5 ±1.6 |
| | | **FI** | 2 ±0.5 | 1.7 ±0.8 | 1.8 ±1.5 |
| **Polymers (µg/cm² CE)** | **shade** | **DI** | 1.1 ±0.07 | 0.4 ±0.1 | 3 ±0.5 |
| | | **FI** | 1.1 ±0.3 | 0.2 ±0.4 | 2.4 ±0.3 |
| | **sun** | **DI** | 1.2 ±0.1 | 0.2 ±0.3 | 0 ±0.0 |
| | | **FI** | 0.7 ±0.5 | 0.5 ±0.2 | 2.9 ±1.3 |

In Riesling the total flavanols increased significantly at pre-veraison and harvest (79 and 89 BBCH, respectively), especially in sun exposed berries (10.8% and 60% respectively) as compared to shade berries (P<0.05) (table 12). Monomers were not detectable at pre-veraison and about 73.3% higher at harvest in sun exposed berries as compared to shade berries (P<0.05) (table 12). Dimers increased significantly at pre-veraison and harvest, especially in sun exposed berries (8.3% and 64.1% respectively) as compared to shade berries (P<0.05) (table 12).The levels of trimers were 23.1% higher at pre-veraison and 90.9% higher at harvest in sun exposed berries as compared to shade berries (P<0.05) (table 12). Polymers increased about 15.8% higher at pre-veraison and 6.9% higher at harvest in sun exposed berries as compared to shade berries (P<0.05) (table 12).

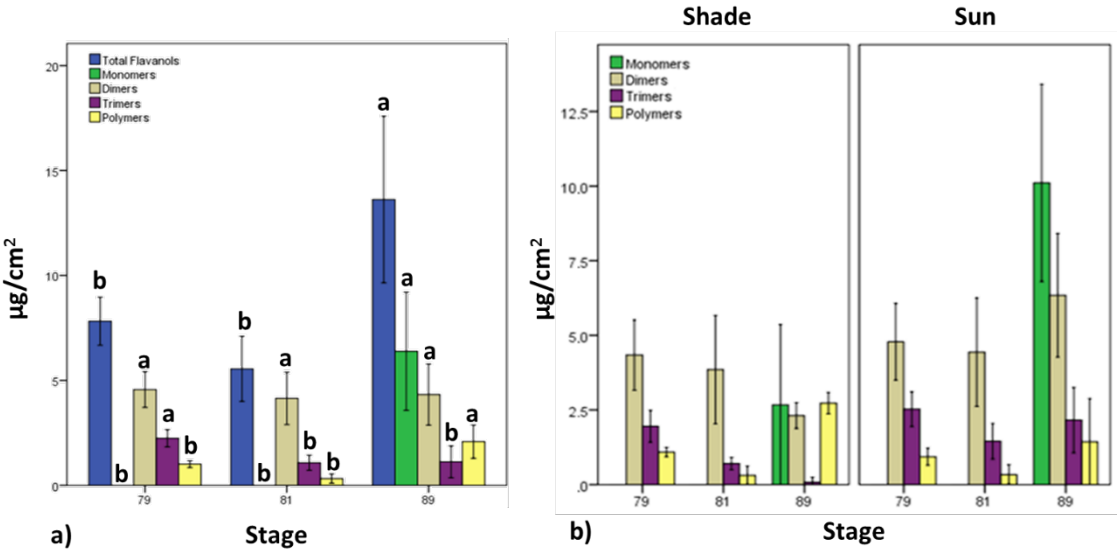


*Figure 22 Riesling: flavanol concentrations (µg/cm²) expressed as catechin equivalent (CE) over the phenological stages (79, 81, 89 BBCH) (a) and considering the exposures (shade and sun) (b). The bars indicate the different flavanol compounds ±standard errors. Total flavanols are expressed as sum of monomers, dimers, trimers and polymers. Different letters within the bars indicate significant differences between the same compound during the three phenological stages at P<0.05.*

In Riesling a general increase during the ripening was found for total flavanols, monomers and polymers. Dimers remained stable, while trimers decreased. The analysis of variance

indicated significant effects attributable to exposure and to phenological stages (fig. 22), but no effects were due to the irrigation regimes ($P<0.05$).

Riesling skin tannins were analysed by spectrophotometer and expressed as mg/berry and mg/g FW (Table 13).

*Table 13 Riesling skin tannins, mean values and standard deviations ($P<0.05$) over the three phenologycal stages (79, 81, 89 BBCH), and considering the two exposure (shade and sun) and irrigation treatments (DI and FI). Data are considered as mg/berry and mg/g FW. The values are espressed as cathechin equivalents.*

| | | | Phenology | | |
|---|---|---|---|---|---|
| **Variable** | **Exposure** | **Irrigation** | **79** | **81** | **89** |
| **Skin Tannins (mg/ berry)** | **shade** | **DI** | 0.12 ±0.04 | 0.12 ±0.08 | 0.29 ±0.02 |
| | | **FI** | 0.11 ±0.03 | 0.15 ±0.02 | 0.15 ±0.01 |
| | **sun** | **DI** | 0.11 ±0.03 | 0.25 ±0.06 | 0.31 ±0.1 |
| | | **FI** | 0.13 ±0.01 | 0.21 ±0.04 | 0.17 ±0.02 |
| **Skin Tannins (mg/g FW)** | **shade** | **DI** | 0.18 ±0.09 | 0.11 ±0.07 | 0.23 ±0.03 |
| | | **FI** | 0.14 ±0.03 | 0.14 ±0.03 | 0.11 ±0.01 |
| | **sun** | **DI** | 0.14 ±0.04 | 0.21 ±0.04 | 0.23 ±0.09 |
| | | **FI** | 0.15 ±0.01 | 0.19 ±0.05 | 0.12 ±0.01 |

Skin tannin concentrations did not changed during ripening, with mean value of about 0.18 (mg/g FW CE) in DI and 0.14 (mg/g FW CE) in FI. However, the phenology was only significant on a content basis, with higher concentrations at harvest ($P<0.05$) (fig. 23). The exposure was not significant. The irrigation treatments were only significant on a per mg/g FW basis, with higher concentrations in DI ($P<0.05$).

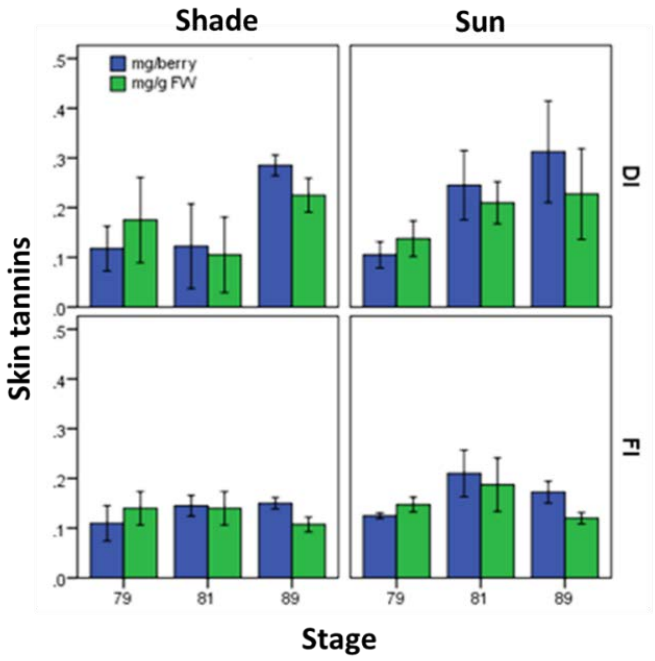


*Figure 23 Riesling: skin tannins showed as content and concentration ±standard errors during three phenological stages (79, 81, 89 BBCH), irrigation (DI and FI) and exposure (sun and shade) treatments.*

In Chardonnay the highest cluster temperatures in the sun was $T_{max}$ = 54°C in both DI and FI. The lowest temperature in the shade was $T_{min}$ = 13°C in both irrigation treatments. The maximum daily temperature amplitude for sun exposed berries was 40°C in FI and 28°C in shade; in DI they were 41°C in sun and 39°C in shaded bunches. In Riesling, the highest cluster temperatures in the sun were $T_{max}$ 54°C in FI and $T_{max}$ 50°C in DI, while the lowest in the shade were $T_{min}$ 3°C in FI and $T_{min}$ 4°C in DI. The maximum daily amplitude temperatures for sun exposed clusters were 47°C in FI and 35°C in shaded; in DI they were 42°C in sun and 40°C in shaded berries.

The number of hours with temperature higher than 40°C and 50°C are described in table 14, as average within the replicates.

*Table 14 Chardonnay and Riesling cluster temperature, measured with Hobo sensors, expressed as sum of hours with temperatures higher than 40 and 50°C.*

| Variety | Exposure | Irrigation | Hours T> 40 °C | Hours T> 50 °C |
|---|---|---|---|---|
| Chardonnay | sun | FI | 116 | 13 |
| | shade | FI | 4 | 0 |
| | sun | DI | 150 | 13 |
| | shade | DI | 82 | 3 |
| Riesling | sun | FI | 86 | 6 |
| | shade | FI | 2 | 0 |
| | sun | DI | 39 | 0 |
| | shade | DI | 41 | 0 |

The hours with temperature higher than 40°C are statistically different between exposure and irrigation treatments in each variety ($P<0.05$). Sun exposed clusters had always higher temperatures, in both irrigation regimes ($P<0.05$).

The average light intensity data are shown in table 15, for both Chardonnay and Riesling.

*Table 15 Chardonnay and Riesling cluster mean (±standard deviation) light intensity (Lux), measured with Hobo sensors, expressed as mean within the exposure and the irrigation treatments.*

| Variety | Exposure | Irrigation | $L_{mean}$ (Lux) |
|---|---|---|---|
| Chardonnay | sun | FI | 25157 ± 45292 |
| | shade | FI | 6847 ± 11055 |
| | sun | DI | 29121 ± 50575 |
| | shade | DI | 14460 ± 30760 |
| Riesling | sun | FI | 36409 ± 63836 |
| | shade | FI | 13994 ± 29047 |
| | sun | DI | 29003 ± 53670 |
| | shade | DI | 21899 ± 48183 |

The light intensity was higher in DI in both varieties with maximum values of about 209424 Lux ($P<0.05$).

## 3.3 Discussion

In the present study, sunlight exposures (sun and shaded treatments) and phenological stages (from 79 to 89 BBCH) directly affected the accumulation patterns of flavonols and flavanols during the ripening in two white grape varieties, Chardonnay and Riesling. A possible effect of two different irrigation regimes (DI and FI) was also considered. Higher concentration of these flavonoid compounds were found especially in sun exposed berry. Quercetin glucoside, quercetin glucuronide and kaempferol-type flavonols, were identified by HPLC analysis. Flavanol monomers, dimers, trimers and polymers were characterized on the basis of their retention time. Tannins were also analysed by spectrophotometer. Temperature and light conditions were studied.

Berry weight increased during ripening, but was not affected by sun exposure in both varieties. In Chardonnay, but not Riesling, the berry weight of the FI regime plants was higher than that in the DI regime. In both Chardonnay and in Riesling total soluble solids increased during ripening, but they were not affected by exposure and/or irrigation treatments. These results agree with other authors' findings (Friedel *et al.*, 2015, Spayd *et al.*, 2002), however they did not test irrigation effects.

In Chardonnay the total flavonols, quercetin glucoside and kaempferol-type flavonols increased during the growing season, with higher values in sun exposed berries at harvest. In Riesling the total flavonols did not change during the ripening, while the quercetin glucoside and kaempferol-derivatives increased over the season, especially in the sun. These results are in accordance with the finding of Friedel *et al.* (2015). In Riesling grapes, these authors found a post-veraison increase in quercetin glycoside in exposed berries, while the total flavonol concentration did not change in artificially shaded treatments nor in the control.

In both cultivars, the increase of quercetin glucoside and kaempferol-type flavonols, suggests an activation of the biosynthetic pathway over the growing season, with a general increase in total flavonols determined by cluster exposure to high light conditions. Also in previous works, flavonol glucosides were higher in the sun, rather than in the

shaded treatment in both red and white varieties (Price *et al.*, 1995, Kolb *et al.*. 2003, Spayd *et al.*, 2002). High temperatures seem to have no effect on flavonol accumulation, in agreement with the finding of other authors (Spayd, *et al.* 2002).

In berries grown in shaded environments, lower flavonol concentrations were detected, but nevertheless they increased from veraison to harvest. These results agree with the finding of Downey *et al.* (2004) and also support their hypothesis of a reduced biosynthesis in shaded berries, rather than a probable degradation during ripening.

In both varieties quercetin-type flavonols dominated the flavonol profile during the ripening, accounting for about 69% of the total flavonols in Chardonnay, and 64% in Riesling. Kaempferol-type flavonols were second in importance, ranging from 9-29% from pre-veraison to harvest, in Chardonnay, and from 15-31%, in Riesling. These outcomes are in accordance with other authors' findings (Castillo-Munoz *et al.*, 2010). In Chardonnay sun exposed berries, quercetin glucoside alone accounted for 55% of total flavonols at harvest, followed by kaempferol-types with 30%. In shaded berries, quercetin glucuronide had the higher contribution at harvest (46% of the total flavonols). In Riesling sun exposed berries at harvest, quercetin glucoside alone accounted for 38% of total flavonols, followed by kaempferol-types with 32% and quercetin glucuronide with 23%. Nonetheless, no increase in quercetin glucuronide concentration took place through the maturation in either variety. It has been shown that, under different environmental stimuli, plants synthesize mostly quercetin-glycosides (Gotz *et al*., 2010, Agati *et al*., 2011, Fini *et al*., 2011), also in accordance with our findings. Different authors suggested a possible explanation for these results. The entire set of genes responsible for the quercetin derivative biosynthesis, and their transcription factors, were already present in liverworts and mosses (Rausher, 2006), and they are early induced by high light, especially UV-B radiation (Falcone Ferreyra *et al.,* 2010), and controlled by changes in cellular redox homeostasis (Heine *et al.,* 2006). In fact, the presence of a catechol group in the *b* ring of the flavonoid skeleton determines a major ability of these flavonols to chelate transition metal ions and to reduce various forms of ROS, as compared with the flavonol or flavone

monohydroxy substituted in the same position (Tattini *et al.,* 2004), plus flavonols absorb in the UV range (Kolb *et al.,* 2003).

In both Riesling and Chardonnay, poor correlations were found between quercetin glucoside and glucuronide (about $r^2<0.30$), corroborating the hypothesis of a possible lack of competition in the biosynthetic pathway between glucosylation and glucuronylation (Castillo-Munoz, *et al.* 2010). No effects on flavonol accumulation were directly due to the irrigation regimes in either cultivar. However, water deficit could have indirectly affected the flavonol accumulation through canopy size reduction and thus, through fruit exposure increase. The variability found in some of the reported data, especially in sun-exposed berries, may partially be due to the different degrees of exposure between and even within single berries. Epidermal UV-screening in leaf, was found to be variable among the same leaf and appears to be increased in response to high UV-B regimes (Caldwell *et al.,* 1983, Flint *et al*., 1985). In the present study, even if the totally exposed and shaded berries were randomly sampled, the spherical shape, no less than its position in the cluster, determined various exposures to sunlight of the same skin tissue and thus diverse localized biosynthesises of flavonols.

HPLC analysis showed a general decrease of Chardonnay flavanols from pre-veraison to harvest, with higher concentration in sun exposed berries than in shaded berries. In Riesling an increase was mostly found in monomers and polymers. The dimers did not change during ripening. Overall, Riesling also showed higher flavanol concentration in the sun. Monomers were absent at 79 and 81 BBCH, in both exposure and irrigation treatments. It could be that their concentrations were not enough to be detected by the HPLC, or they were lacking at pre-veraison and veraison. In a recent work on Riesling, flavanol accumulation was found not to be affected by either shading or leaf removal (Friedel, *et al.* 2015). Riesling ripening occurred later in the season, as compared to Chardonnay; Riesling was harvested 24 days after Chardonnay. Thus the temperature and light conditions were different. Even if the temperatures were still very high before harvest, however, they were lower for Riesling than for Chardonnay. The average light

intensity in Riesling was higher, and in general the canopies were less vigorous, as compared to Chardonnay. It is difficult to separate the effects of temperature and sunlight on berry composition, since most of the biochemical pathway are affected by both of them. Nonetheless, it could be possible that higher light exposure microclimate stimulated a functional protection mechanism that involved monomers and polymers (which increased over the season), as well as a potential stimulating effect on polymerization of flavanols. Some authors explain the increase in polymers throughout the season as result of a decrease in plant vigour (Cortell, *et al.* 2005), with indirect effects on bunch exposure. In the studied cultivars, Chardonnay had much higher flavanol concentration, than Riesling. However, even if the results regarding flavanol accumulation were discordant, similar patterns were found with respect to light exposure. In each phenological stage, the higher flavanol amount was always found in sun exposed berries. In previous work, it was hypothesised that light only affects flavanol accumulation if exposure treatments are applied at flowering (Koyama, *et al.* 2012). Moreover, some authors attribute the variability within cultivars to a potential association of grape proanthocyanindins with polysaccharides (Bindon *et al*., 2010b, Bindon *et al.*, 2010a), or also their modification by oxidation (Poncet-Legrand *et al.*, 2010) or subcellular partitioning (Gagne *et al.*, 2006).

For what concerns the analysis of skin tannins performed by spectrophotometer, the concentrations were close to those stated by other authors in different varieties (Liang, *et al.* 2011, Harbertson, *et al.* 2002). Bearing in mind that condensed tannins are a heterogeneous mixture of different polymer sizes and subunit compositions (Haslam, 1998), their efficacy for protein precipitation was found to rise with increasing degrees of polymerization (Harbertson, *et al.* 2014). Thus, skin tannins measured with the spectrophotometer should only be compared with polymer results obtained using the HPLC method. In Chardonnay, skin tannins expressed as mg/g FW, showed the same decreasing trend as the polymers detected by HPLC. For Riesling the stage was not significant when the results were expressed in mg/g FW, whereas the same trend as the polymers was found on mg/berry bases. In both varieties, the irrigation treatment was

significant only if the skin tannins were expressed as mg/g FW, for Chardonnay a reasonable cause could be the different berry size, which was bigger in FI. The exposure was significant in Chardonnay, whereas this was not so in Riesling. In 2014, the Riesling canopy was heterogeneous and less vigorous, as corroborated from the similar light conditions between the irrigation and exposure treatments; these conditions might have indirectly affected the overall exposure of berries, and thus the results.

Under similar light conditions the difference in temperature within the two irrigation treatments in the sun, could be due to the less vigorous canopy of DI plants compared with FI plants, which overall lead to greater cluster exposure, in both varieties. Nonetheless, the temperatures were always lower in FI and higher in DI, supporting the hypothesis of a possible no (or little) effect on flavonol biosynthesis. Shaded-bunch temperatures were always lower as compared to sun exposed ones, as direct solar heating did not occur in the shaded-conditions. These results agree with other authors' finding (Friedel *et al.*, 2015, Spayd *et al.*, 2002). Hot temperatures did not reduce or inhibit the formation of flavonols in either cultivar.

# Chapter 4 General Discussion

In white grapes, photosynthetic pigments play important roles in berry color and environmental interactions (e.g. irradiation stress). In the Italian experimentation, new specific reflectance indices for the evaluation and estimation of photosynthetic pigments were proposed on the basis of grape berry reflectance spectra. These new equations are also able to discriminate between chlorophyll *a* and *b*. Indeed, the wavelengths of major interest for their absorption detection were identified. The formulas proposed were based on the Gaussians half heights. If chlorophyll quantifications were achieved directly from reflectance spectra, for carotenoids the absorption bands did not allow good reflectance correlations. Nonetheless, thanks to the physiological relation of photosynthetic pigments, the chlorophyll/carotenoid ratio was used to estimate carotenoid content. Because their proportion changes during berry development, the index coefficients can be adapted in relation to the BBCH phenological stage. These indices demonstrated good correlations with the destructive quantifications.

Also, the degradation intensity of the chlorophyll was different from that of the carotenoid during maturation, leading to a change in their absorption proportion throughout ripening. This finding allows suggesting that in white berries, the colour change during berry development is not related to the activation of a specific biosynthetic pathway, but is mostly the result of catabolic processes. Even so, the flavonols were not studied in this part of the research.

Chardonnay and Riesling showed different susceptibility to sunburn. The results suggest that for each variety, the timing of leaf removal during the day is fundamental to reducing the appearance of brown color in the berry skin. In Chardonnay it would be better to avoid any leaf removal especially in pre-veraison, in the morning. Because Riesling was more susceptible during the afternoon, it would be recommendable to remove leaves, if necessary, during the early morning.

The intent of the American research was to study the effect of cluster exposure on fruit composition, over the ripening in Chardonnay and Riesling. Soluble solids, flavonols and flavanols were studied. Two irrigation treatments were also considered, but no direct effects were found, except when speaking of tannins measured by spectrophotometer analysis and expressed in amount per berry. Nonetheless, the irrigation regime could have indirectly affected cluster microclimate, influencing the flavonoid biochemical pathways in the skins. Indeed, water stress could have affected the vigour of the canopy, generating an effect on bunch shading and/or exposure to light and, consequently, affecting the temperature of the berries. Soluble solids increased over the season, but were not affected by either exposure or irrigation treatments. Berry weight was not affected by light exposure. In Chardonnay the FI treatment increased berry size. Flavonols increased during ripening, especially in the sun. Indeed, their concentrations were much higher in sun exposed berries than in shaded berries. Under sun exposure in both varieties, the most produced flavonols were the quercetin-types. In general Chardonnay berries had slightly higher concentrations of flavonols than Riesling berries, especially at harvest. However, keeping in mind that the concentration of these compounds is affected by the degree of cluster illumination (Downey *et al.*, 2004), flavonol concentrations cannot be considered characteristic of the cultivar. The amount of flavanol monomers, dimers, trimers and polymers was greater in sun exposed berries than in shaded berries. Unlike in Chardonnay, in Riesling monomers were not detected by HPLC during the pre-veraison and veraison.

High light regimes potentially stimulated a protection mechanism in the skin, increasing flavonols over the season. Since flavanols also had highest concentration in the sun, it could be speculated a possible involvement of these molecules in the photo-protection mechanisms of the berry. The spectral maximum absorption band of flavanols, around 280 nm, lets one hypothesize a potential involvement of these compounds in UV-screening, potentially enhancing the UV-B shielding (UV-B band 280-315 nm), as it was demonstrated for hydroxycinnamic acid (see Kolb *et al.*, 2003).

Hot temperature did not affect flavonols biosynthesis, but could have had a role in significantly reduced flavanol formation in Chardonnay, especially at harvest. Actually, due to the precocity of Chardonnay variety as compared to Riesling, the berries developed under higher temperature regimes in 2014. Though, in Chardonnay the flavanols content was much higher than in Riesling, but this difference might be characteristic of the variety. In Riesling the increase of flavanols over the season, expecially regarding the monomers and polymers at harvest, could be related to high light conditions, and lower maximum day with hot temperatures, as compared to Chardonnay. Riesling was harvested almost 25 days later. A possible explanation for these results could be an involvement of sunlight in stimulating flavanol polymerization. Grape berries exposed to sunlight had visual evidence of sunburn, but the percentage of cluster exposure were not tested in this work.

From a scientific stand point it could be interesting to separate the effects of the light and temperature on the biosynthesis of flavonoid in white grape berry. Canopy measurement and percentage of cluster exposure determinations, in respect to the diverse irrigation regimes, should be tested in relation to sunburn appearance on berry skins.

From a wine making stand point, these results suggest that cluster exposure generates greater amounts of compounds that potentially elicit bitterness and astringency in the final wine. Leaf area reduction as consequence of water deficit, thus irrigation regime management, seems to indirectly affect the flavanols and flavonols by providing high exposure of fruit, also generating sunburn appearance. Thus, to avoid late and unpleasant disappointment, trellis systems that have excessive cluster exposure and/or several leaf removal procedures should be avoided, especially in hot temperature and high light intensity macroclimate zones.

# Chapter 5 Conclusions

Chlorophylls and carotenoids play important roles in berry colour and eco-physiology. The reflectance indices are non-invasive methods which allow a fast data collection during the short phenological period of interest. The new formulas, as well as the published index performances, presented in this work, could give alternative methods to support further studies concerning cultivar classification and/or physiological roles in abiotic stresses. However, the main limitation of this reflectance-based approach is related to the difficulties in data elaboration but, nevertheless, it provides noninvasive information at high resolution and multiple high density observations on the same sample. Moreover, costs are usually reasonable. The susceptibility to berry sunburn in pre-veraison and veraison appeared influenced by the daily time of leaf removal, affecting differently the two considered cultivars, Chardonnay and Riesling.

The compositional differences between Chardonnay and Riesling were highlighted in the American results. In both cultivars flavonol and flavanol accumulations were affected by sunlight exposure, during the growing season. The lack of monomers in Riesling in the early stages of ripening, if confirmed with next year's data, could be an interesting starting point to study the biosynthetic connection within monomers and polymers, since the hypothesis about this topic is still hazy.

In future experiments it could be interesting to combine the study of the role of photosynthetic pigments under high light and temperature conditions, together with the accumulation of flavonoids in berry skin. Also it could be interesting to separate the effects of light from those of temperature on the biosynthesis of these compounds. Also separate vinification of grapes harvested from shade and sun exposed clusters, under different irrigation regimes, could be a point to support both growers and winemakers, in terms of canopy and water stress management, and thus berry composition affecting the final wine and its style.

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