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Effect of the plant sink/source balance on the metabolic content of the *Vitis vinifera* L. red grape

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Abstract

Despite their low concentration, secondary metabolites are essential to the organoleptic quality of grapevine fruit. Anthocyanins of the fruit skin cells are the principal components of the pigmentation of the red grape. Glycosylated aroma precursors (GAPs), i.e. alcohols, C_{13}-norisoprenoids, phenols and terpenes determine the aroma potential of the juice and the resulting wine. The regulation of the sink/source (S/S) balance is considered as a one of the more powerful tools to adapt grape composition to technological objectives. In this study, we have manipulated the S/S of *Vitis vinifera* varieties and quantified the changes on the accumulation of secondary metabolites at the arrest of phloem unloading in the grape. The results demonstrated that the manipulation of the S/S doesn't de-correlate the accumulation of secondary versus primary metabolites. Decreasing S/S drastically limited the accumulation of either primary metabolites (till -70%), anthocyanins (till -70%) and GAPs (till -81%) per plant, with a huge production shortfall of molecules of interest per cultivated area unit.

Keywords: *Vitis vinifera*, fruit, sink/source, metabolites, anthocyanins, glycosylated aroma precursors.

1. Introduction

The grape is a fleshy, non-climacteric fruit exhibiting a double sigmoid of growth (Coombe, 1976). After a first phase of green growth, berries soften (Coombe, 1984) and phloem unloading shifts from symplasmic to apoplasmic pathway (Zhang et al., 2006), triggering a sudden acceleration of sugars

import and a second phase of water import, known as ripening (Matthews et al., 1987). When the berry is at the maximum of water and solutes contents, phloem unloading stops and berries shrivel. During grape development concentration in metabolites evolve as a function of the balance between metabolite biosynthesis and growth-related dilution (Ojeda et al., 2002; Dai et al., 2011; Bigard et al., 2018, 2019). A range of primary and secondary metabolites contribute to the quality of the red grape, such as sugars, quantity and balance between tartaric and malic acids, anthocyanin pigments and aroma compounds. Organic acids are reaching a maximum level per berry just before the onset of ripening (Bigard et al., 2019). During ripening, sugars increase from 50-100 mmol/l to 1 mol/l at ripe stage, mainly in the form of hexoses (Famiani et al., 2014). In *V. vinifera* varieties, anthocyanins are accumulated to a few hundreds of mg/l (Cheynier, 2005; Yang et al., 2014). Grapes mainly contain 3-O-glucoside derivatives of malvidin but also other forms, i.e. delphinidin, cyanidin, petunidin, peonidin and pelargonidin, are also present but in smaller proportions (Hugueney et al., 2009; Pantelić et al. 2016). Aroma precursors are very important contributors to the wine grape quality (Dariet et al., 2012; Alem et al., 2018). Terpenes, C_{13}-norisoprenoids, phenols and non-terpenic alcohols are the most abundant aroma compounds to be accumulated as volatile (free) or non-volatile and glycosylated (bounded) forms. The non-volatile glycosylated aroma precursors (GAPs) represent 80-90% of the aroma potential in most of *V. vinifera* varieties (Sanchez-Gomez et al., 2017; Alem et al., 2018).

There is a range of viticultural practices influencing grapevine development (cover-grassing, irrigation…) and/or manipulating the microclimate to improve the accumulation of primary and secondary metabolites in the grape (Reynolds, 1989; Alem et al., 2018). Climatic factors, such as temperature or light, regulate grape production and composition through complex effects at plant and organ levels (Sanchez and Dokoozlian, 2005; Carbonneau et al., 2020; Alcántara-Novelli Dias et al., 2019). Several approaches, such as winter pruning, shoot or leaf removal, shoot trimming or cluster thinning can be implemented to control the photosynthetic potential and the yield and regulate the balance between the sink and the source (S/S) for photoassimilates (Huglin, 1957; Champagnol, 1984; Smart et al., 1990; Sukje et al., 2013; Eltom et al., 2015). S/S is also regulated in other perennial fruit crops to stimulate fruit growth and improve ripening and fruit quality (Link, 2000). For instance, fruit thinning resulted in higher sugar contents in sweet cherry (Whiting and Lang, 2004), apple (Solomakhin and Blanke, 2010), plum (Seehuber et al., 2011) and pear (Lopez et al., 2011). Bunch thinning is also recommended in Viticulture (Carbonneau et al., 1977; 2020) to improve wine grape composition but the results are still controversial (Alem et al., 2018).

The methods used in previous studies is the first limitation to interpret the effects of S/S on grape composition. Indeed, the phenotyping was monitored through solute concentration and not with

accumulation parameters (Carbonneau et al., 1977; Rescic et al., 2015; Song et al., 2018). Unfortunately, in the absence of accurate physiological landmarks, concentration and accumulation effects can be confused. Indeed, during ripening the concentration is driven by the ratio between the biosynthesis and water import but, when berry shriveling replaces phloem unloading, solute concentrations become dependent on water loss (Bigard et al., 2018; 2019). Another limitation of previous studies is the focus on concentration gains, while the potential losses of metabolite per cultivated area have not been assessed. Based on in-field experiments, the aim of this study was to revisit the effect of the manipulation of the S/S on the accumulation of the main primary and secondary metabolites in the grapevine fruit. Sugars, organic acids, glycosylated anthocyanins and aroma precursors were selected as the main sink for the non-structural carbon in grape. For the first time, the physiological ripe stage was targeted to allow accurate comparisons in quantity of metabolites at the arrest of solute import. Another originality of this work lies in the expression of the variables to: i) describe possible metabolic arbitration keys and ii) perform quantitative assessments at plant level.

2. Materials and methods

2.1 Plant material and growing conditions

Experiments were performed with *V. vinifera* cv. Syrah and Cabernet Sauvignon at the INRA experimental centre of Pech Rouge, South of France (43°8'35.180" Lat, 3°7'57.442" Long). Syrah and Cabernet Sauvignon were respectively planted in 2001 and 2004 and grafted on 140Ru and SO4 rootstocks. The planting geometry of the experimental plots was 2.5x1.00 m. Plants were trained by Guyot pruning and canopy managed by vertical shoot positioning. Climatic data were collected during the 3 years of experiment (**Figure S1** and **Table S2**). For each treatment, plant water deficit was weekly monitored by measuring predawn (Ψ_b) leaf water potential (Carbonneau et al., 2004; Taylor et al., 2012) and drip irrigation managed to keep Ψ_b > -0.7 MPa.

2.2 S/S treatments and determination of the fruit sampling date

Each variety was experimented in a separated plot. Three rows from the border of the experimental plots and 3 plants from the beginning of each row were excluded from the experiments to avoid border-effects. For each combination of treatment/genotype/year, 3 blocks of 3 plants were randomly selected. The controls corresponded to the plots managed through standard practices to target a of 1.5-2 kg of grapes per vine (5.5-7 t/ha). Every year of experiment, 2 level of S/S were compared for each variety: in 2015 and 2016, a control (as a higher S/S) and a modality with a lower S/S; in 2017, a control (as a lower S/S) and a modality with a higher S/S. For treatments targeting low S/S, bunches were thinned to 50% (2015) and 70% (2016) before the onset of fruit ripening,

when berries reached pea size. For treatments targeting high S/S (2017), the number of buds per plants were doubled during winter pruning. S/S balance was estimated at posteriori as described by Ravaz (1912), i.e. calculating the ratio between fresh fruit weight and winter pruning biomass.

The strategy of sampling was determined to target the end of phloem unloading in the fruit, a transitory stage corresponding to the time when berry volume is maximum (Bigard et al., 2019). Two or 3 weeks after the onset of ripening, for each variety and year, the volume of 2 reference bunches was weekly and non-destructively monitored by Archimed's method as described by Torregrosa et al. (2008). This allowed the anticipation of the growth slowing down period (Bigard et al., 2018) for a precise determination of the time of the maximum level of water and biomass accumulation in the fruit. Consequently, the date of sampling was customized depending on the variety and the year (**Table S2**).

2.3 Sample preparation and metabolite analyses

At the sampling date, all the fruits of each treatment were collected separately (approximately 10 - 40 kg of grape per treatment). Then, within each lot, bunches were divided into 5 or 6 portions. Bunches portions were carefully mixed, to make sampling as representative as possible. Two samplings were performed separately in triplicates. First, 200 berries were randomly picked up for sugars, acids, and anthocyanins determination, and immediately analyzed. Then, 2 kg per treatment was collected for GAPs determination. These samples were stored at -20 °C till analysis.

2.3.1 Primary metabolites

Each repetition of the 200 berries was weighed and grinded at room temperature with a domestic blender for 2 minutes at maximum speed. An aliquot of 2 ml of the clear juice was immediately prepared on which main sugars (glucose and fructose) and organic acids (tartaric and malic acids) were analysed as in Bigard et al. (2019). The contents in primary metabolites were expressed in mass, moles or equivalents of moles of C per volume, organ or plant, considering hexoses and organic acids respectively carry 6 and 4 atoms of carbon.

2.3.2 Anthocyanins

For each lot of 200 berries, 50 g of the crude extract were analysed as in Bigard et al. (2019). Anthocyanins were expressed in mass, moles or equivalents of moles of C per volume, organ or plant. Since anthocyanin monomers present in *V. vinifera* red fruit, i.e. pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin display very close C skeleton (He et al., 2012) and malvidin is the most abundant (Gao et al., 1997; Kallithraka et al., 2005), the calculation was

performed in malvidin equivalent to finally determine the equivalents in moles of C (17 carbons per molecule).

2.4.3 GAPs

GAPs were quantified according to the method described by Schneider et al. (2001). Briefly, 500 g of defrosted berries (one night at 4 °C) were crushed with a domestic blender at room temperature and centrifuged at 7000 rpm (20 min, 10 °C). Three hundred fifty ml of clear supernatant were sampled and added with 17.5 g of PVPP. After filtration, the sample was aliquoted in 3x100 ml to constitute analytical triplicates. Glycosidic fraction was extracted using C18 cartridges (500 mg). The bound glycosidic fraction was recovered by a final elution with 10 ml methanol.

The glycosidic fraction was dried with air flux in a water bath (40 °C), and then hydrolysed in a phosphate citrate buffer (sodium hydrogen phosphate 0.2 M, citric acid 0.1 M, pH 5.0) using a glycosidasic enzyme preparation (Rapidase revelation Aroma, Oenobrands, France). The aglycons released were then extracted using pentane/dichloromethane (2/1; v/v). After concentration and addition of 4-nonanol as internal standard, the extract was analysed using GC-MS in full scan mode. The compounds identified were semi-quantified and classified into 4 families: terpenes, alcohols, phenols and norisoprenoids (**Table S3)**.

GAPs were expressed in mass, moles and equivalents of moles of C per volume, organ or plant. To convert GAPs into equivalents of moles of C, the molecular mass of each type of GAPs was weighted according to their abundance and considering aglycone and glycoside structure. For GAPs displaying both mono- and di-glycosylated structures, an average of both molecules C number was used. All the calculations were performed at the level of the molecule's families: alcohols, C_{13}-norisoprenoids, phenols and terpenes.

2.5 Data analyses and graphic representations

Experiments were carried on a randomized block design, with three repetitions for each treatment. All statistical analyses were performed using the software package INFOSTAT® (University of Cordoba, Argentina). The data were subjected to analysis of variance (ANOVA). Mean comparisons were performed using Fisher's least significant difference (LSD) test and significance was set at $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***). Principal component analysis (PCA) was performed to identify the overall behavior of the experiment.

3. Results

The experiment having been carried out over a period of 3 years with 2 genotypes. The first part of the results (**3.1**.) presents the inter-annual fluctuations of vegetative and fruit developments of the

control plots and the varietal specifications of the grape composition. In a second part of the results, the effects of the manipulation of the S/S ratio on the accumulation of primary and secondary metabolites (**3.2**), as well as the correlations between variables (**3.3**) are detailed.

3.1 Effect of the genotype and the environment on plant development and fruit composition

Huglin index (Huglin, 1978) and PET showed some stability between the growing seasons (**Figure S1, Table S2**). However, temperature and rainfall varied during the ripening, with higher T° and rainfall in 2015 in comparison to 2016 and 2017 (**Figure S1**). Despite these environmental fluctuations, genotypic differences in the fruit ripening timing were obvious with earlier sampling dates for Syrah than Cabernet Sauvignon.

For both varieties, yield and vegetative development remained in line with the other plots of the experimental center (data not shown). Depending on the year of experiment, Syrah (**Figure 1**) fruit yield ranged from 2100 to 2730 g/plant and stem biomass from 619 to 630 g/plant, displaying year-to-year phenotypic stability. Yielding 3930 g/plant of fruits in 2015, twice as high as in 2017, Cabernet Sauvignon showed a greater sensitivity to the environment.

These behavior differences resulted in S/S variations in the control plots (**Table 1**). Depending on the year, S/S varied between 3.4 and 4.4 and 2.8 to 6.9 for Syrah and Cabernet Sauvignon respectively, with a genotypic significant effect only in 2015 and highly significant GxE interaction every year. The volume of the berry was the most stable variable with no statistical differences between varieties and year. All primary metabolites varied depending on the genotype and the year with statistically significant GxE interactions. For both varieties, the level of accumulation of the primary metabolites was of the same magnitude as usually observed for other *V. vinifera* varieties. For instance, sugars ranged from 174 to 195 g/l (0.97 to 1.08 mol/l) for Syrah and from 189 to 217 g/l (1.05 to 1.21 mol/l) for Cabernet Sauvignon, with a glucose/fructose ratio around 1. Tartaric acid was the major organic acid found in ripe grapes, with malic acid being 30 to 50% less. The sum of the 2 organic acids ranged from 59 to 84 mmol/l in Syrah and from 53 to 87 mmol/l in Cabernet Sauvignon, the lowest values being observed in 2015 for both genotypes.

The accumulation of secondary metabolites fluctuated depending on the variety and the year (**Table 2**). For anthocyanins, both varieties displayed a similar range of accumulation either expressed in concentration or in quantity per berry or plant with statistically significant effects of the year. In 2015, anthocyanins were accumulated at much lower concentration and quantity per fruit compared to the 2 following years in both varieties. For GAPs, no specific variations were observed for each family of aroma precursors studied, which showed a similar behavior to the total GAPs in the different years (**Table S4**). With 77 % of the total GAPs in average, glycosylated alcohols were the

more abundant GAPs, with other groups being less than 10 %. We observed a high correlation between the quantities of each aroma precursor's family and the total GAPs expressed either in concentrations in the fruits or in quantities per plant (**Table 3**). Therefore, to simplify the representation of the GAPs data set, only total GAPs were considered and further discussed in this manuscript. Fruit GAPs concentration depended on the variety and the environment, with a significant effect of the genotype and GxE interactions (**Table 2**). This was also true when GAPs were expressed in quantity per fruit or per plant, suggesting a higher dependence of this parameter to environmental fluctuations. Compared to anthocyanins which ranged from 0.68 to 1.30 g/l (0.035 to 0.066 mol/l), GAPs concentrations were about 1000 times less., 0.58 to 1.12 mg/l (0.026 to 0.054 mmol/l).

Comparing the abundance of metabolites in equivalents of moles of carbon allows a different representation of their proportions in the ripe grape (**Table 4**). With variation from 6 to 7.5 equivalents of moles of C, sugars and organic acids are largely the major fractions of the non-structural C of the grape with little difference across genotypes and years. Secondary compounds were more subject to fluctuations, with 50% and 90% of variations of anthocyanin concentration depending on the year in Syrah and Cabernet Sauvignon respectively. Similarly, GAPs showed large fluctuations, up to 80% between years for Syrah and Cabernet Sauvignon with no relationship with anthocyanin contents (R= 0.06). When expressed in equivalents of moles of C, GAPs exhibited concentrations a thousand times lower than anthocyanins. Indeed, the ratio GAPs/anthocyanins varied from 0.39 10^{-3} to 1.34 10^{-3} depending on the year and the variety. As anthocyanins, GAPs represent a very low fraction of the non-structural C of the fruit, i.e. 3.4 to 8.9 10^{-6} compared to sugars depending on the year and the variety.

3.2 Effect of the manipulation of S/S on the accumulation of metabolites in the grape

We have modulated fruit load levels to establish several S/S balances during the 3 years of experiments. Bunches or bud load manipulation significantly impacted total fruit weight per plant in all years and varieties, with a maximum of harvest in 2015 for Cabernet Sauvignon and a minimum of harvest in 2016 for Syrah (**Figure 2**). The S/S fluctuations, that are the results of the variation of both fruit load and canopy volume, were found highly statistically significant for each variety and year. Inverse proportionality between fruit load and canopy development can be observed for both varieties whatever the year, but these differences are only statistically significant in 2017 for Cabernet Sauvignon.

Despite the huge variations of S/S, berry volume remained constant (data not shown). Thus, to simplify the presentation of the data sets, data are only presented in concentration and in quantity

per plant, but not in quantity per fruit. For primary metabolites, except in 2015 with Syrah, higher S/S tended to decrease the concentration of sugars in the fruit at ripe stage, but the effect was not very consistent and only statistically significant in 2016 and 2017 with Syrah and in 2017 with Cabernet Sauvignon (**Table 5**). Conversely, when expressed in quantity per plant, S/S was highly correlated with the level of sugar accumulation with a very high level of statistical significance whatever the year and the variety. In the high S/S modality, accumulation of sugars per plant increased from 120% (2015) to 231% (2016) for Syrah and from 115% (2016) to 142% (2015) for Cabernet Sauvignon. For organic acids, no consistent links between S/S and concentration were observed. On the other hand, the effects of S/S on their accumulation per plant were very high. At high S/S, accumulation of organic acids per plant increased from 97% (2015) to 362% (2016) for Syrah and from 133% (2016) to 182% (2015) for Cabernet Sauvignon. Sugars and organic acids exhibited the same response to S/S with a coefficient of correlation 0.74 (p-value 0.041) between the variations of sugars and organic acids depending on S/S.

Statistical analyses did not show any statistically significant effect of the S/S on the concentration in anthocyanins of the ripe grape (**Table 6**). Conversely, when expressed in quantity per plant, S/S was well correlated with the anthocyanin contents, with high statistically significant effects whatever the year and the variety. At high S/S, the accumulation of fruit anthocyanins per plant increased from 85% (2015) to 232% (2016) for Syrah and from 120% (2016) to 190% (2015) for Cabernet Sauvignon. For GAPs, no clear links between S/S and the contents in GAPs could be evidenced (**Table 6**). Indeed, GAPs concentrations increased at low S/S in 2015 and 2016 with a statistical significance in 2015, but the contrary was observed in 2017. Similar observations could be done with Cabernet Sauvignon. When expressed in quantity per plant, S/S effects were found very significant. Except with Syrah in 2015, at high S/S, GAPs accumulation per plant increased from 254% (2016) to 437% (2017) for Syrah and from 151% (2016) to 218% (2015) for Cabernet Sauvignon. We did not find a consistent correlation between the variations of anthocyanins and GAPs in relation with S/S (R=0.47, p-value 0.15).

The ratios between primary and secondary metabolites accumulated in the ripe grape (**Figure 3**) showed a great stability across years for each variety. In comparison to primary metabolites, the sum of anthocyanins and GAPs represented 1.2 to 2% for Syrah (**Fig. 3A**) and 1 to 1.5% for Cabernet Sauvignon (**Fig. 3B**). This confirms that, regardless of the S/S, Syrah tended to accumulate relatively more secondary metabolites than Cabernet Sauvignon**.** Except in 2015 for Syrah, S/S variations did not significantly change the ratio between primary and secondary metabolites in the ripe fruit.

3.3 Correlation between the variables

Principal component analysis (PCA), with all variables expressed in concentration was performed to graphically illustrate the overall behavior of the experiment (**Fig. 4A**). The first principal component (PC1, 36.4% of total variance of the experiment) showed clearly the differences between the two varieties. Syrah samples, located on the right of figure, were mainly characterized by a higher content in total GAP berries, mainly phenols, terpenes and alcohols than the Cabernet Sauvignon samples (to the left of **Fig. 4A**), more rich in sugars. The second principal component (PC2, 34.3% of the total variance of the experiment) mainly explains the difference between years. Actually, samples from 2016 and 2017 (located at the bottom of **Fig. 4A**) displayed higher contents in anthocyanins, tartaric and malic acids than in 2015, regardless of the variety considered. Finally, for each variety/year situation, the different sink/source ratios (S/S+ and S/S-) were placed very close, showing a rather modest impact of this factor on the whole experiment in terms of berry composition. However, when variables were expressed in quantity per plant (**Fig. 4B**), the PCA showed that the main factor explaining the overall behavior of the experiment (PC1, 68,7%) was clearly the S/S ratio. The highest S/S values are invariably placed to the right of the figure, with higher primary and secondary components contents, regardless of the variety or the year. Major families of GAPs presented similar fluctuations of concentration when S/S is manipulated and no relationship with anthocyanins (**Fig. 4A)**. In terms of metabolites to be accumulated per plant (**Fig. 4B)**, S/S was a strong driver of the response, with all primary and secondary metabolites (except the C_{13}-norisoprenoids) showing the same behaviors as confirmed by the matrix of correlation (**Table 3**).

4. Discussion

This study was based in 2 *V. vinifera* varieties producing anthocyanin-pigmented fruits, which were selected because of their biological behaviors. Syrah, originated from Rhone Valley (France), is an early ripening variety, with a low vegetative vigor and well adapted to hot Mediterranean climates. Cabernet Sauvignon, originated from Bordeaux region (France), is a late ripening variety with a high vegetative vigor, adapted to moderate-temperature climates.

Yearly fluctuations of the climate are one the main difficulties to perform in field experiments because of GxE interactions. According to the multicriteria classification of the vine growing regions (Tonieto and Carbonneau, 2004), during experiments, climatic conditions were typical to semi-arid Mediterranean areas. We have implemented precise specifications to regulate the vegetative and reproductive development according to the varietal characteristics and the agro-pedological situations of each plot. Despite a stable Huglin index and PET, some variations of temperature and rainfall during fruit ripening could explain observed E effects (Jones and Davis, 2000; Schmidtke et al., 2020). Indeed, the regime of temperature during spring can impact the bud

fruitfulness and flowering set (Guilpart et al., 2014); whereas water regime determines fruit growing capacity (Ojeda et al., 2002). During ripening, temperature can also influence the balance between primary metabolites (Rienth et al., 2016) and the accumulation of anthocyanins (Mori et al., 2007) and other secondary metabolites (Torregrosa et al., 2017; Blancquaert et al., 2019). The night temperature of the 30-day period before harvest plays an important role in regulating the metabolism of the plant, mainly in relation to secondary metabolites (polyphenols and aroma compounds) in grapes (Tonietto and Carbonneau, 2004). Within the three years of experiments, 2015 was characterized by the warmest minimum temperatures during the 30 days before grapes sampling and these conditions are known to be unfavorable for grape aromas and pigmentation (Hoschberg et al., 2015; Spayd et al. 2002; Wu et al., 2019).

Some berry composition parameters of the control plots could appear to be slightly different from the ones observed in commercial vineyards (Schmidtke et al., 2020). For instance, Syrah generally provides wines with 14% of ethanol (i.e. 245 g/l or 1.36 mol/l of sugars) while, in our experiments control plots ranged between 174 g/l (0.97 mol/l) and 196 g/l (1.09 mol/l) of sugars. Cabernet Sauvignon is classically harvested around 230 g/l of sugars (1.28 mol/l), while the contents of control plots ranged between 189 g/l (1.05 mol/l) and 217 g/l (1.21 mol/l) of sugars. These differences can be explained by the strategy to determine ripe fruit stage. In commercial vineyards, wine grapes are harvested after the phloem discharge has stopped (Schmidtke et al., 2020) to concentrate anthocyanins and decrease tannin astringency. This delays the harvest to higher sugar contents in the grapes. In this study, we targeted the moment when phloem discharge stops in the fruit, i.e when water and solute quantities are maximal (Bigard et al., 2018). The level of primary metabolites accumulated in the control samples analysed in this study, i.e. around 1 mol/l of sugars and 40 mmol/l of tartaric acid are typical for fruit sampled at this stage (Bigard et al., 2019). In this study, the ratio between glucose and fructose and the tartaric acid concentration were exactly found as expected for a *V. vinifera* ripe fruit (Bigard et al., 2019).

Analysing the effect of S/S on the accumulation of the different metabolites of the fruit, needs relevant variables to represent the trophic competition for photoassimilates. Indeed, sugars, organic acids or secondary metabolites display a huge diversity of structures. In a first approximation, we converted the molar concentration of each family of compounds with their average number of carbon atoms. For main sugars and organic acids, the conversion is accurate as major molecules of these families have similar C skeletons. For anthocyanins, we have expressed the concentration in moles of malvidin and then used the structure of the malvidin to get the equivalent of moles of C. For GAPs, which is a very complex family (Dariet et al., 2012; Alem et al., 2018), we also made a calculation considering the specificity of the components of each type of compounds analyzed. This

conversion system, which is surely questionable from a strict chemical point of view, allowed the comparison of the C mobilised per each metabolic family. Sugars and organic acids were found to be the major destination of the non-structural C in the grapevine fruit. Secondary compounds, which represent only a small fraction of the photoassimilates allocated to the fruit, were shown more influenced by the genotype and the year than primary metabolites.

The S/S balance was appreciated through the Ravaz index (Ravaz, 1912). In grapevine, vegetative vigor and lateral branching is dependent on the number of buds determined by winter pruning (Champagnol, 1984). Because winter pruning eliminates most of the biomass of the year's shoots, the weight of winter pruning wood is well correlated with the volume of the canopy during the season (Smart et al., 1990; Keller, 2015). An excess of fruits due to bud overloading can lead to plant exhaustion due to an excessive S/S (Howell, 2001). Depending on its intensity, bunch thinning can induce a significant reduction of the yield (Carbonneau et al., 1977; Dokoozlian and Hirschfelt, 1995; Di Profio et al., 2011; Rescic et al., 2015). The Ravaz index incorporates all the factors modifying the vegetative growth of the main and secondary axes and the volume of the yield (Shinkis and Vance, 2013; Carbonneau et al., 2020).

Despite the huge variations of experimented S/S, for both varieties, the volume of the berry remained stable in agreement with previous reports (Rescic et al. 2015; Bogicevic et al. 2015; Wang et al., 2018). This is due to the late date of bunch thinning which avoided compensation effects on grape growth. This option was taken because, for wine grapes, it is commonly admitted that fruit growth should not be encouraged to avoid metabolite dilution due to the massive importation of water associated with ripening (Carbonneau et al., 1977; Dokoozlian and Hirschefelt, 1995). Conversely, in most of the other perennial crops, fruit thinning is generally performed early after the fruit set to limit the trophic competition and maximise fruit size and ripening (Barone et al., 2014; Costa-Vizzotto, 2000; Guardiola and Garcia-Luis, 2000).

As Song et al. (2018), who studied the effect of bunch thinning with Cabernet Sauvignon in the Weibei (China), we did not observe significant changes in sugars and organic acids concentrations related to S/S. These results disagree with some former reports (Carbonneau et al. 1977; Di Profio et al., 2011; Rescic et al. 2015). The discrepancies of the results about the effect of S/S on primary metabolites could be due to the differences in sampling strategy (as discussed above) or to environmental specificities. Indeed, depending on the region and the year of experiment, potential of C assimilation varies as a function of the level of photosynthetic active radiation (PAR), water supply and temperature (Chaves et al., 2010). Thus, reducing crop load can result in a range of situations in terms of S/S balance and physiological responses of the plants.

We also did not observe a huge impact of S/S on the evolution of organic acid contents. Both major organic acids, i.e. tartaric and malic acids are accumulated during the first growing phase to a peak just before the onset of ripening (Bigard et al., 2019). During green growth, there is a low level of competition for photoassimilates because organic acids are only accumulated to a few hundred mmol/l while sugars stay below 100 mmol/l in the green fruit (Bigard et al., 2018). During ripening, both organic acids are diluted by fruit growth, while malic acid is also metabolised at the onset of sugar phloem discharge (Rienth et al., 2016; Bigard et al., 2018). This suggests the lack of variations in organic acid concentration regardless of S/S level is linked to the little changes in berry volume. These observations are consistent with those of Song et al. (2018) who observed, depending on the year and the variety, a very small or no effects of bunch thinning on organic acid concentration in the grape. Nevertheless, at plant level, S/S was found strongly linked to the quantity of sugars and organic acids allocated to the fruit. A reduction of S/S could decrease up to half of the primary metabolites accumulated in the fruit leading to a significant loss of production per cultivated area unit.

The effects of S/S manipulation on the concentration of GAPs were similar to the one observed with primary metabolites. Conversely to Di Profio et al. (2011), Rescic et al. (2015) and Song et al. (2018), we did not observe a significant difference in the concentration in anthocyanins related to S/S. However, due to the complexity of the synthesis of these compounds, which is highly dependent on the cluster microclimate (Haselgrove et al., 2008), and considering the possible effects of concentration and degradation during fruit shrivelling (Rio Segade et al., 2008; Rescic et al., 2015; Bigard et al., 2019), it does not seem reasonable to compare our results with previous reports which did not target a precise physiological stage.

Results did not show clear links between the level of S/S and the concentration of GAPs in the ripe grape. This study is the only one that aimed to analyse the impact of S/S on the accumulation of GAP compounds in the grapevine fruit (Dariet et al., 2012; Alem et al., 2018). Song et al. (2018) analysed during 2 years the impact of bunch thinning on the concentration of aroma volatile molecules at technological ripening. As observed here with GAPs, they found variable effects depending on the year, the variety and the compound. According to the year, S/S effects were not or only hardly statistically significant but with inconsistent trends of variation. This shows the difficulty to experiment with compounds that are accumulated at a very low level in strong interaction with environmental factors (Suklje et al., 2019; Schmidtke et al., 2020).

On the other hand, when expressed in quantity per plant, both anthocyanidins and GAPs were shown strongly related to the S/S level. Indeed, at high S/S, the increase reached 232% for anthocyanins and 437% for GAPs in Syrah, and 190% for anthocyanins and 218% for GAPs in Cabernet

Sauvignon, with a very high level of statistical significance. Another interesting observation is in relation to primary versus secondary partitioning under S/S fluctuations. Despite GAPs and anthocyanins include sugar moieties, which represent a large part of their molecular weight, they represent a very small proportion of the non-structural C biomass of the fruit in comparison to primary metabolites (1 to 2% depending on the year and the variety). In this study, whatever the level of S/S applied, we could not de-correlate the balance between primary and secondary metabolisms. This demonstrates that the manipulation of the plant C balance is not efficient in modifying metabolic pathway homeostasis to improve the contents in secondary metabolites regardless of primary compounds.

In the ripe grape, GAPs remain a very small fraction (approx. 1‰) of the C mobilised within secondary metabolites. This represents a very low metabolic cost for the plant in comparison to the massive flow of major organic osmotica, i.e. sugars and organic acids which are associated with grape ripening. In this study, we demonstrated that attempting to install a more comfortable S/S balance at plant level does not increase GAPs concentrations in the ripe grape. Other cultivation practices able to modify metabolic pathways without significantly modifying global plant C balance or hempairing yield performances would be more profitable for commercial vineyards. Among these practices, those aiming to improve grape's microclimate (Smart et al., 1985; Jackson and Lombard, 1993; Zoecklein et al. 1998; Dariet et al. 2012), to control plant water status (Ojeda et al., 2002; van Leeuwen et al., 2009; Acevedo-Opazo et al., 2010) or to spray biostimulants (Sanchez-Gomez et al., 2017) have long been shown to be effective to increase the contents in secondary metabolites.

5. Conclusion

Grape composition depends on complex interactions between plant physiology and environmental factors, but also technological objectives. A range of plant management practices are implemented to tune S/S ratio to improve the composition of the ripe fruit. As the concentration of fruit solutes evolve through either import or biosynthesis mechanisms but also by water content variation, or combining both, their quantification is only accurate when associated with a precise physiological monitoring. This study was designed to not confuse the accumulation of organic solutes in the fruit, which occurs after phloem unloading stops, with their concentration during shriveling. We observed a very low and/or inconsistent impact of S/S on the concentration of either primary and secondary metabolites in the ripe grape. In contrast, the manipulation of S/S strongly impacted the yield and the amount of major qualitative metabolites accumulated per plant. Altogether these observations suggest that cluster or bunch thinning should be considered with circumspection considering the loss of molecules of interest that this entails per cultivation area unit.

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Legends of the figures

Figure 1 - Fresh fruit (external ring) and annual shoot biomass (internal ring) production (g/plant) during the 3 years of experiments for *V. vinifera* cv. Syrah and Cabernet Sauvignon.

Figure 2 - Variations in fresh fruit weight, annual shoot biomass and sink/source ratio (S/S) obtained by fruit load manipulation in *V. vinifera* cv. Syrah and Cabernet Sauvignon. (ns) non-significant.

Figure 3 - Effect of sink/source manipulation on the balance between secondary (Anthocyanins+GAPs) and primary (Sugars+organic acids) metabolites in *V. vinifera* cv. Syrah (A) and Cabernet Sauvignon (B). (ns) non-significant.

Figure 4 - Principal component analyses of grape composition variables at ripe stage for two *V. vinifera* varieties analysed together. Data, obtained after modulating sink/source ratios during 3 years of experiment, are expressed in concentration per fresh fruit unit (A) and in quantity per plant (B). S/S+ (full symbols) and S/S- (empty symbols) refer to the level of source/sink in Syrah (Sy, circular symbols) and Cabernet Sauvignon (CS, square symbols) varieties. The vectors indicate the direction and the weight of each variable in the 2 selected axes.

Table captions

Table 1 - Effect of the genotype (G) and the environment (E) on S/S balance and primary metabolite accumulation in the ripe grape of *V. vinifera* cv. Syrah and Cabernet Sauvignon in the control plots. Means +/- standard deviation (SD). (nd) not determined, (ns) non-significant.

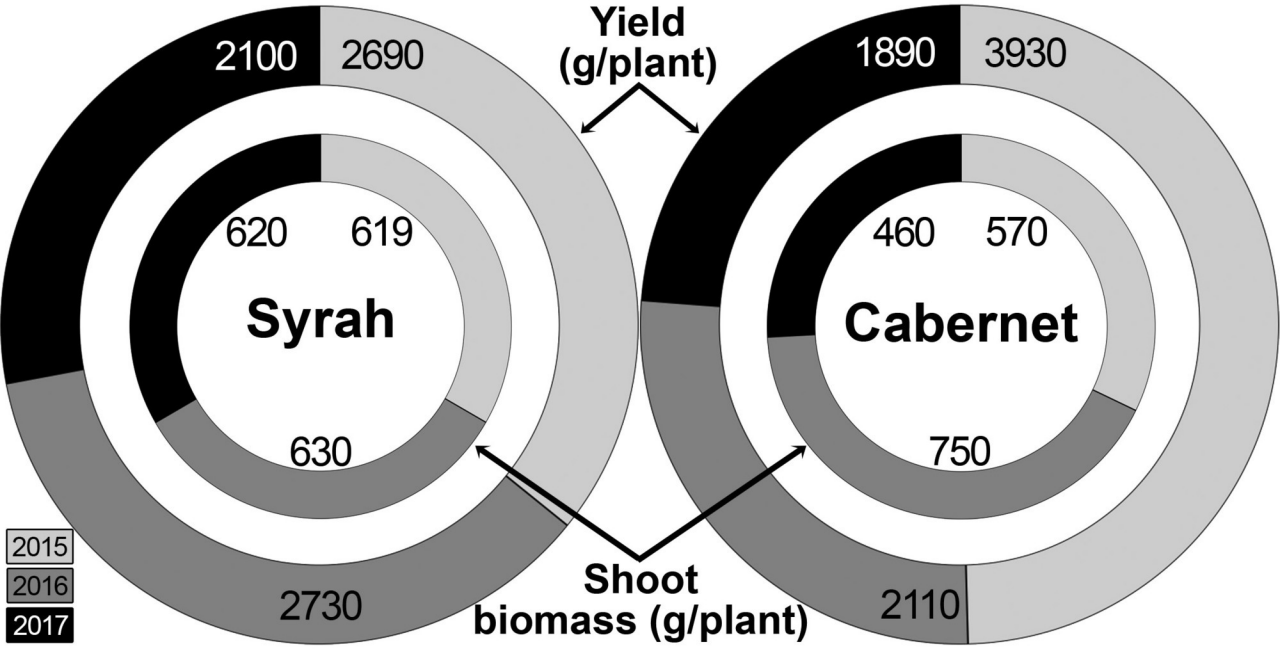
Table 2 - Effect of the genotype (G) and the environment (E) on S/S balance and secondary metabolite accumulation in the ripe grape of *V. vinifera* cv. Syrah and Cabernet Sauvignon in the control plots. Means +/- standard deviation (SD). (ns) non-significant.

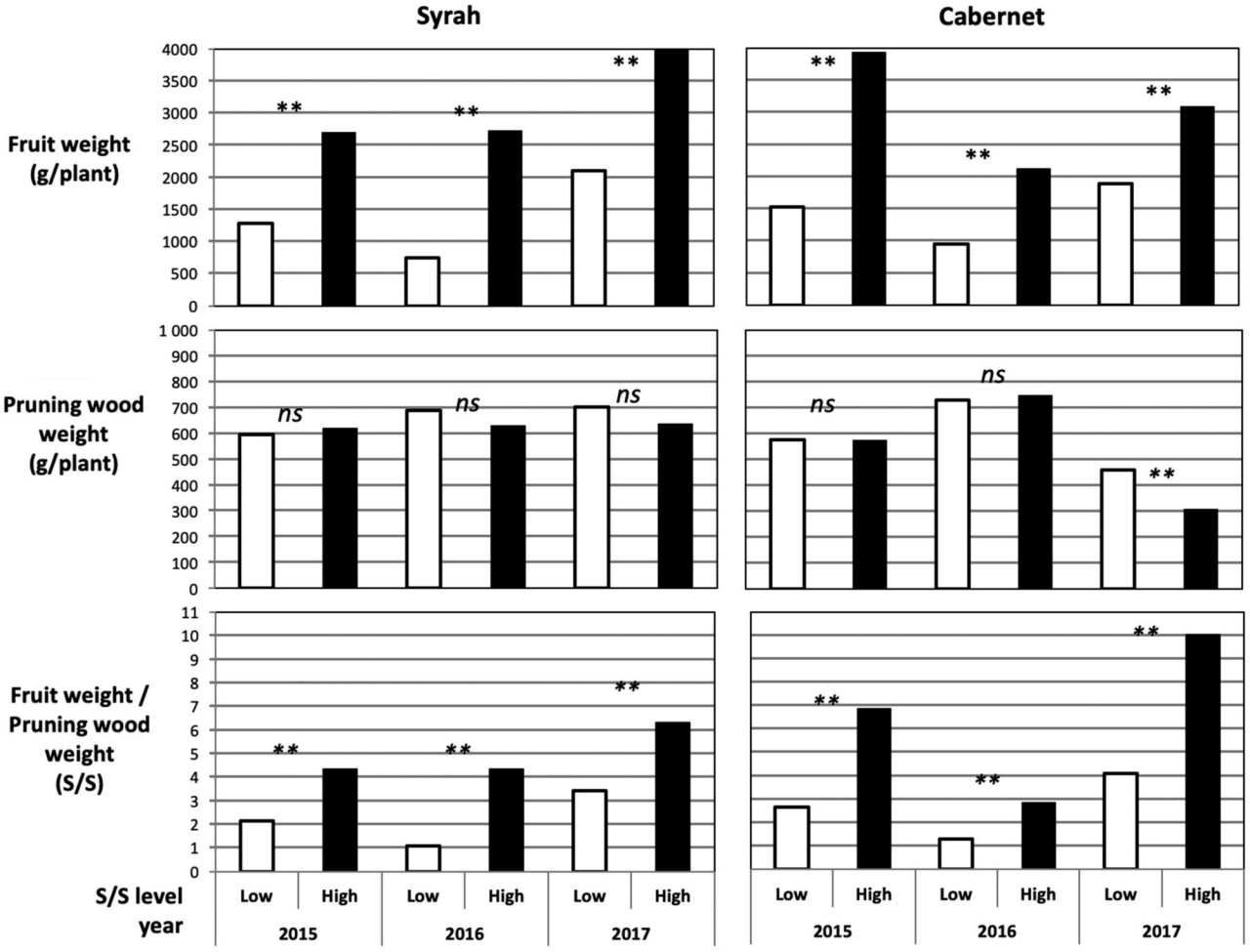
Table 3 - Matrix of the correlations between the family of GAPs present in the ripe grape of *V. vinifera* cv. Syrah and Cabernet Sauvignon. Variables are expressed in g/l and mg/plant, all years and S/S levels being analysed together.

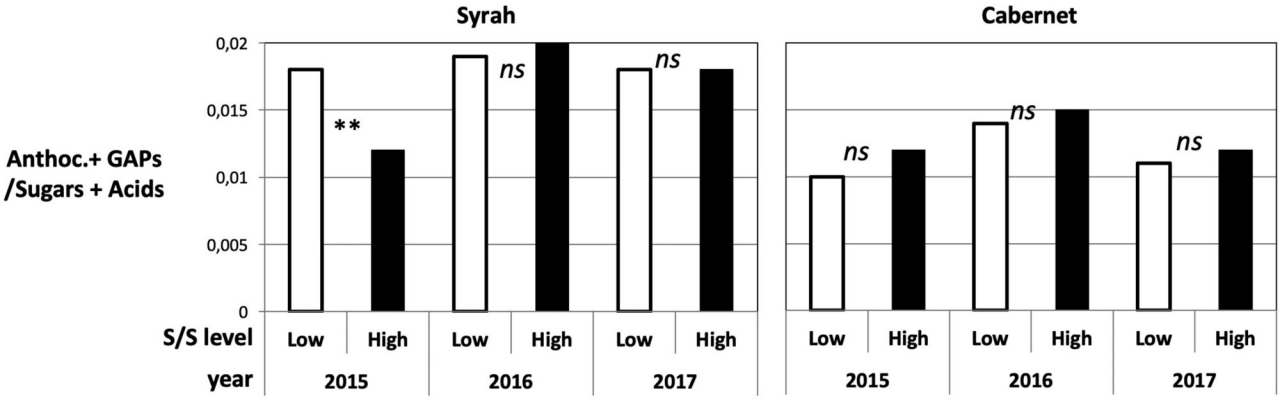
Table 4 - Variation of metabolite contents in the ripe grape of *V. vinifera* cv. Syrah and Cabernet Sauvignon in the control plots, expressed in C equivalent. Means +/- standard deviation (SD).

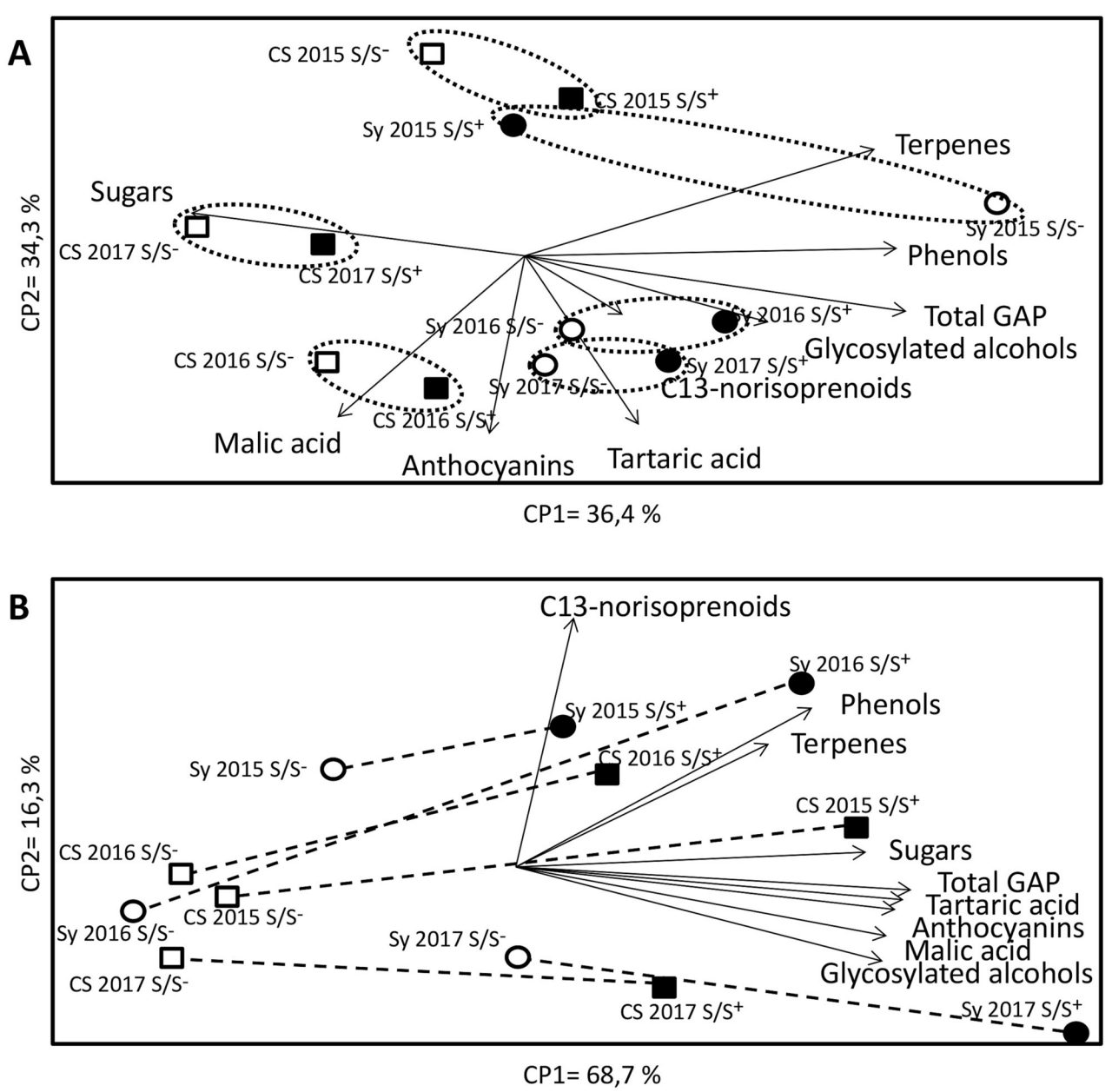
Table 5 - Impact of S/S on primary metabolite accumulation in the ripe grape and at plant level for *V. vinifera* cv. Syrah and Cabernet Sauvignon. Means +/- standard deviation (SD). (ns) non-significant.

Table 6 - Impact of S/S on secondary metabolite accumulation in the ripe grape and at plant level for *V. vinifera* cv. Syrah and Cabernet Sauvignon. Means +/- standard deviation (SD). (ns) non-significant.


Yield
(g/plant)
2100
2690
620
619
Syrah
630
1890
3930
460
570
Cabernet
750
2015
2016
2017
2730
Shoot
biomass (g/plant)
2110


Syrah
Cabernet
Fruit weight (g/plant)
Pruning wood weight (g/plant)
Fruit weight / Pruning wood weight (S/S)
S/S level
year
Low
High
2015
2016
2017
**
ns


Syrah
Cabernet
Anthoc.+ GAPs /Sugars + Acids
0,02
0,015
0,01
0,005
0
**
ns
ns
ns
ns
ns
S/S level
Low
High
Low
High
Low
High
Low
High
Low
High
Low
High
year
2015
2016
2017
2015
2016
2017


A
CS 2015 S/S-
CS 2015 S/S+
Sy 2015 S/S+
Terpenes
Sugars
Sy 2015 S/S-
CS 2017 S/S-
CS 2017 S/S+
Phenols
Sy 2016 S/S-
Sy 2016 S/S+
Total GAP
Glycosylated alcohols
CS 2016 S/S-
Sy 2017 S/S+
Sy 2017 S/S-
C13-norisoprenoids
CS 2016 S/S+
Malic acid
Anthocyanins
Tartaric acid
CP2= 34,3 %
CP1= 36,4 %
B
C13-norisoprenoids
Sy 2016 S/S+
Phenols
Sy 2015 S/S+
Terpenes
Sy 2015 S/S-
CS 2016 S/S+
CS 2015 S/S+
Sugars
CS 2016 S/S-
Total GAP
Tartaric acid
CS 2015 S/S-
Anthocyanins
Sy 2016 S/S-
Sy 2017 S/S-
Malic acid
Glycosylated alcohols
CS 2017 S/S-
CS 2017 S/S+
Sy 2017 S/S+
CP2= 16,3 %
CP1= 68,7 %

Variable	Year (E)	Genotype (G)				Statistics			
		Syrah		Cabernet		G	E		G X E
		Mean	*SD*	Mean	*SD*		Syrah	CS	
S/S balance	2015	**4.4**	*0.5*	**6.9**	*0.4*	**	ns	***	***
	2016	**4.3**	*1.0*	**2.8**	*0.2*	ns			
	2017	**3.4**	*0.6*	**4.1**	*0.6*	ns			
Berry volume (ml/berry)	2015	**1.7**	*0.3*	**1.4**	*0.1*	ns	ns	ns	ns
	2016	**1.5**	*0.1*	**1.5**	*0.1*	ns			
	2017	**1.3**	*0.1*	**1.3**	*0.1*	ns			
Sugars (g/l)	2015	**195**	*7*	**189**	*9*	**	*	*	***
	2016	**180**	*5*	**200**	*3*	*			
	2017	**174**	*4*	**217**	*1*	***			
Sugars (g/berry)	2015	**0.33**	*0.01*	**0.26**	*0.01*	**	***	*	***
	2016	**0.26**	*0.01*	**0.30**	*0.1*	*			
	2017	**0.23**	*0.01*	**0.29**	*0.1*	***			
Sugars (g/plant)	2015	**479**	*17*	**675**	*31*	***	***	*	***
	2016	**454**	*17*	**390**	*7*	***			
	2017	**340**	*8*	**371**	*9*	***			
Glucose (mmol/l)	2015	**541**	*19*	**541**	*19*	*ns*	ns	***	**
	2016	**517**	*26*	**568**	*14*	*			
	2017	**518**	*9*	**641**	*2*	***			
Fructose (mmol/l)	2015	**524**	*24*	**524**	*24*	*ns*	*	***	**
	2016	**491**	*24*	**548**	*13*	*			
	2017	**479**	*8*	**613**	*3*	***			
Tartaric acid (mmol/l)	2015	**44**	*3*	**35**	*1*	**	***	***	***
	2016	**47**	*1*	**52**	*1*	**			
	2017	**52**	*1*	**46**	*2*	*			
Malic acid (mmol/l)	2015	**15**	*2*	**18**	*1*	ns	***	***	***
	2016	**27**	*2*	**35**	*3*	*			
	2017	**32**	*1*	**30**	*1*	**			

Variable	Year (E)	Genotype (G)				Statistics			
		Syrah		Cabernet		G	E		G X E
		Mean	*SD*	Mean	*SD*		Syrah	CS	
Anthocyanins (g/l)	2015	**0.85**	*0.04*	**0.68**	*0.13*	ns	***	**	ns
	2016	**1.28**	*0.04*	**1.30**	*0.18*	ns			
	2017	**1.28**	*0.05*	**1.29**	*0.20*	ns			
Anthocyanins (mg/berry)	2015	**1.42**	*0.07*	**0.94**	*0.18*	*	****	**	*
	2016	**1.87**	*0.06*	**1.92**	*0.27*	ns			
	2017	**1.68**	*0.07*	**1.73**	*0.26*	ns			
Anthocyanins (g/plant)	2015	**2.08**	*0.11*	**2.43**	*0.47*	ns	***	ns	*
	2016	**3.24**	*0.11*	**2.53**	*0.23*	**			
	2017	**2.50**	*0.10*	**2.21**	*0.34*	ns			
GAPs (µg/l)	2015	**696**	*102*	**1017**	*96*	*	**	*	***
	2016	**1223**	*111*	**975**	*126*	ns			
	2017	**1016**	*64*	**580**	*12*	*			
GAPs (µg/berry)	2015	**1.17**	*0.17*	**1.41**	*0.13*	ns	*	**	**
	2016	**1.78**	*0.16*	**1.45**	*0.19*	ns			
	2017	**1.34**	*0.08*	**0.78**	*0.02*	*			
GAPs (µg/plant)	2015	**1711**	*250*	**3637**	*345*	**	**	***	***
	2016	**3046**	*226*	**1903**	*287*	*			
	2017	**1987**	*126*	**991**	*20*	**			

Syrah					
µg/l mg/plant	Alcohols	C13-Nor.	Phenols	Terpenes	**Total GAPs**
Alcohols		0.69	0.23	0.21	**0.89**
C13-Norisop.	0.91		0.89	0.78	**0.84**
Phenols	0.59	0.91		0.95	**0.54**
Terpenes	0.49	0.82	0.97		**0.52**
Total GAPs	**0.95**	**0.96**	**0.83**	**0.74**	

Cabernet Sauvignon					
µg/l mg/plant	Alcohols	C13-Nor.	Phenols	Terpenes	**Total GAPs**
Alcohols		0.57	0.61	0.58	**0.86**
C13-Norisop.	0.87		0.57	0.90	**0.46**
Phenols	0.93	0.74		0.51	**0.84**
Terpenes	0.91	0.96	0.80		**0.43**
Total GAPs	**0.98**	**0.68**	**0.88**	**0.75**	

Variable	Year	Genotype			
		Syrah		Cabernet	
		Mean	*SD*	Mean	*SD*
Sugars Eq C (mol/l)	2015	6.5	*0.2*	6.3	*0.3*
	2016	6.1	*0.3*	6.7	*0.2*
	2017	6.0	*0.1*	7.5	*0.1*
Organic acids Eq C (mmol/l)	2015	235	*11*	211	*1*
	2016	295	*6*	349	*14*
	2017	336	*4*	304	*7*
Anthocyanins Eq C (mmol/l)	2015	44	*2*	35	*7*
	2016	66	*2*	67	*9*
	2017	66	*3*	66	*10*
GAPs Eq C (µmol/l)	2015	30	*9*	47	*4*
	2016	54	*28*	43	*6*
	2017	46	*3*	26	*3*
GAPs/Sugars (10^-6)	2015	4.6	*0.6*	7.5	*1.1*
	2016	8.9	*0.4*	6.3	*0.9*
	2017	7.6	*0.2*	3.4	*1.9*
GAPs/Org. A (/10^6)	2015	129	*17*	219	*22*
	2016	176	*2*	122	*14*
	2017	137	*7*	107	*5*
GAPs/Antho. (10^-3)	2015	0.68	*0.01*	1.34	*0.01*
	2016	0.82	*0.01*	0.64	*0.01*
	2017	0.70	*0.05*	0.39	*0.02*

Variable	Year	Genotype								
		Syrah					Cabernet			
		Low S/S			High S/S		Low S/S			High S/S
		Mean	SD		Mean	SD	Mean	SD		Mean	SD
Fruct + gluc (mol/l)	2015	1.04	*0.09*	ns	1.08	*0.04*	1.13	*0.08*	ns	1.05	*0.05*
	2016	1.12	*0.02*	*	1.01	*0.05*	1.15	*0.01*	ns	1.12	*0.03*
	2017	1.10	*0.01*	**	1.01	*0.01*	1.31	*0.01*	***	1.25	*0.01*
Fruct + Gluc (mol/plant)	2015	1.21	*0.10*	***	2.66	*0.10*	1.55	*0.10*	***	3.75	*0.17*
	2016	0.77	*0.01*	***	2.55	*0.12*	1.01	*0.01*	***	2.17	*0.05*
	2017	1.19	*0.01*	***	3.74	*0.03*	1.41	*0.01*	***	3.36	*0.01*
Tartaric + malic acids (mmol/l)	2015	63	*1*	ns	59	*3*	49	*2*	*	53	*1*
	2016	72	*1*	ns	74	*2*	84	*2*	ns	87	*3*
	2017	78	*1*	ns	78	*1*	67	*1*	ns	68	*1*
Tartaric + malic acids (mmol/plant)	2015	73	*2*	***	144	*7*	67	*2*	***	189	*1*
	2016	50	*1*	***	186	*4*	73	*2*	***	170	*6*
	2017	85	*1*	***	292	*1*	72	*1*	***	183	*2*

Variable	Year	Genotype								
		Syrah					Cabernet			
		Low S/S			High S/S		Low S/S			High S/S
		Mean	SD		Mean	SD	Mean	SD		Mean	SD
Anthocyanins (mmol/l)	2015	50	*6*	ns	44	*2*	31	*5*	ns	35	*7*
	2016	73	*13*	ns	66	*2*	67	*5*	ns	67	*9*
	2017	93	*2*	ns	61	*1*	60	*4*	ns	59	*4*
Anthocyanins (mmol/plant)	2015	58	*5*	***	107	*5*	43	*7*	**	125	*24*
	2016	50	*5*	***	166	*5*	59	*4*	**	130	*18*
	2017	101	*3*	***	228	*3*	65	*5*	***	157	*12*
GAPs (µmol/l)	2015	75	*27*	**	29	*9*	39	*11*	ns	47	*4*
	2016	54	*12*	ns	52	*2*	38	*6*	ns	43	*6*
	2017	40	*5*	**	62	*5*	32	*5*	ns	40	*7*
GAPs (µmol/plant)	2015	78	*7*	ns	74	*10*	53	*15*	***	169	*10*
	2016	37	*6*	***	131	*4*	33	*6*	**	83	*11*
	2017	43	*4*	***	231	*13*	34	*4*	**	107	*19*