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Disertação de Mestrado
Mestrado em Biologia Molecular Biotecnologia e Bioempreendedorismo em Plantas

Trabalho efectuado sob a orientação do
Prof. Doutor Hernâni Gerós
e do
Doutor José Eiras Dias

outubro de 2013

DECLARAÇÃO

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Título da Tese de Mestrado:

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Ano de conclusão: 2013

Designação do Mestrado:

Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Universidade do Minho, ____/____/_____

Assinatura: _____

This work was supported by European Union Funds (FEDER/COMPETE-Operational Competitiveness Programme) and by national funds (FCT-Portuguese Foundation for Science and Technology) under the project FCOMP-01-0124-FEDER-022692 and the research project PTDC/AGR-ALI/100636/2008. This work was also supported by the European project INNOVINE (ref. 311775), and from the networking activities within the European funded COST ACTION FA1106 “QualityFruit” and COST Action FA1003 “East-West Collaboration for Grapevine Diversity Exploration and Mobilization of Adaptive Traits for Breeding”.

Acknowledgments

To my supervisor, Prof. Hernâni Gerós, who granted me the opportunity to work in his group where I hope to continue for some time! I have learned a lot from him. A sincere thank you.

To Eng. Eiras Dias for his co-orientation and for providing the samples from Dois-Portos.

To Margarida Santos and João Brazão from INIAV for providing the samples from the South region, and to Eng. Teresa Mota from EVAG for providing the samples from the North region.

A special thanks to the members of our small group: Viviana, Henrique, Richard and Artur always willing to help. Thank you for your companionship.

To Prof. Ana Cunha, who we appealed to whenever statistics doubts, among others, appeared.

To my laboratory colleagues, Joana and Rose, as well as all the others.

To Bé who taught to me much more than Portuguese.

To my family without highlighting or forgetting anyone because all contributed to my unexpected journey. A heartfelt thanks.

Para ser grande sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes, ...
Ricardo Reis

Abstract

Mature berries of grapevine (*V. vinifera* L.) have thousands compounds including water, sugars, phenolics, organic acids, amino acids and mineral salts. This complex composition depends on many and diverse factors including the choice of grape variety and rootstock, edaphoclimatic conditions and agricultural practices, like canopy management and irrigation, among others. The present study aimed to evaluate how edaphoclimatic conditions affect the metabolomic profile of the grapes from three Portuguese varieties - Alvarinho, Arinto and Padeiro de Basto – sampled in 2012 season in two distinct ampelographic collections, at North (Estação Vitivinícola Amândio Galhano - EVAG; Arcos de Valdevez) and South (Instituto Nacional de Investigação Agrária e Veterinária – INIAV; Dois-Portos) Portugal. In parallel, it was aimed to study the genotype-dependent metabolomic profile upon comparison the grape metabolome from the three above-mentioned varieties cultivated in the same the region. From October 2011 to October 2012, the air temperature and the evapotranspiration values in the south were consistently higher than in the north where a higher amount of precipitation was associated to higher relative humidity and soil water content. To study the metabolome, lyophilized samples were analysed qualitatively by GC-TOF-MS at the Genome Center (UC Davis - USA) and free amino acids quantification was performed by an Amino acid Analyser system by Ansynth Service B.V. (Netherlands). Results showed that the growing region clearly affected the metabolome profile of the grape berries. For instance, mature grapes from Alvarinho contained much less tartaric and malic acids in the south than in the north, and grape berries from the three varieties cultivated in the north were much richer in amino acids than in the varieties from the south. In addition, several cultivar-dependent traits were identified in the present work. For instance, sorbitol and proline were very abundant in grapes from Padeiro de Basto and Tinto Cão, respectively. In parallel experiments, twenty-one varieties cultivated in Portugal were selected to assess in mature grape berries the varietal dependence of phenolic content and antioxidant capacity, and proline content. Berries from the Tinto Cão red variety showed the highest proline concentration followed by berries from the white variety Airén. The concentration of total phenolics in mature grape berries from red varieties was higher than in white varieties. Borraçal grapes reached values as high as 5.0 μg/mg FW. Additional studies were performed to evaluate how grape berry metabolism is affected in grapevines infected with Leafroll-associated virus-3. In infected Cabernet Sauvignon glucose and fructose in the berry were reduced over the control, by 19% and 17%, respectively, while the concentration of sucrose did not change from clean to infected plants. Contrarily, in grape berries from Touriga Nacional there was a significant reduction in sucrose concentration by 40% in infected plants, while glucose and fructose concentration did not change from clean to infected plants. The expression of the sugar transport genes VvHT1 and VvHT6 was also studied in response to virus infection, together with the activity of metabolic enzymes. The results of the present study are compared with other metabolic studies performed so far in grape berries from other cultivars and discussed in the context of the ongoing climate changes.

Resumo

Bagos de uva (*Vitis vinifera* L.) na fase madura possuem milhares de compostos diferentes, incluindo água, açúcares, compostos fenólicos, ácidos orgânicos, aminoácidos e sais minerais. Esta composição complexa depende de diversos factores, incluindo o tipo de cultivar e de porta-enxertos utilizados, as condições edafoclimáticas e as práticas agrícolas, como a poda e o tipo rega, entre outros. No presente estudo pretendemos avaliar o perfil metabólico de bagos de uva de três cultivares portugueses - Alvarinho, Arinto e Padeiro de Basto – amostrados em 2012 de duas coleções ampelográficas localizadas no norte de Portugal (Estação Vitivinícola Amândio Galhano - EVAG; Arcos de Valdevez) e no Sul (Instituto Nacional de Investigação Agrária e Veterinária – INIAV; Dois-Portos). Em paralelo, pretendeu-se estudar a influência do genótipo no perfil metabólico, quando se comparou o metaboloma do bago das três variedades referidas cultivadas na mesma região. Entre outubro de 2011 e outubro de 2012, os valores de temperatura do ar bem como da taxa de evapotranspiração no sul foram consistentemente maiores do que no norte, onde uma maior taxa de precipitação estava associada com valores maiores de humidade relativa e de conteúdo de água no solo. Para estudar o metaboloma, as amostras liofilizadas foram analisadas qualitativamente por GC-TOF-MS (Genome Center; UC Davis - USA) e os aminoácidos livres foram quantificados por um Analisador de Aminoácidos (Ansynth Service; B.V.- Holanda). Os resultados mostraram que o tipo de região afectou significativamente o perfil metabólico dos bagos de uva. Por exemplo, bagos maduros de Alvarinho continham níveis de ácido tartárico e málico muito inferiores no sul do que no norte, e bagos de uva das três castas cultivadas no norte eram muito mais ricos em aminoácidos do que das castas cultivadas no sul. Adicionalmente, no presente trabalho foram identificadas várias características dependentes do cultivar. Por exemplo, os níveis de sorbitol e de prolina foram muito superiores em bagos de Padeiro de Basto e Tinto Cão, respetivamente. Em experiências paralelas foram selecionadas 21 castas cultivadas em Portugal para avaliar em bagos maduros o conteúdo em compostos fenólicos e o potencial antioxidante, bem como os níveis de prolina. Em bagos de uva do cultivar Tinto Cão foi medido o valor mais elevado de prolina, a que se seguiu o valor obtido em bagos da variedade Airén. Em bagos maduros das castas tintas foram medidos níveis mais elevados de fenólicos totais do que nas castas brancas. Em bagos da variedade Borraçal foram medidos valores tão elevados quanto 5,0 μg/mg peso fresco. Foram desenvolvidos estudos adicionais preliminares para avaliar o metabolismo do bago em videiras infectadas pelo vírus do enrolamento. Na casta Cabernet Sauvignon os níveis de glucose e frutose no bago foram inferiores em 19 e 17%, respectivamente, aos medidos em plantas saudáveis, enquanto que os níveis de sacarose não sofreram alteração. Ao contrário, em bagos de uva de plantas infectadas de Touriga Nacional observou-se uma redução significativa da concentração em sacarose de 40% enquanto que as concentrações de glucose e de frutose não variaram. Após infecção com o vírus do enrolamento foram também estudados nos tecidos do bago os níveis de transcritos dos transportadores de açúcares VvHT1 e VvHT6, bem como a atividade de algumas enzimas. Os resultados do presente trabalho são comparados com outros estudos sobre o metabolismo do bago desenvolvidos em outros cultivares e são discutidos no contexto das variações climatéricas em curso.

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Abbreviations and acronyms

ABC	ATP-Binding Cassette Transporters
ABCC1	ATP-Binding Cassette, sub-family C, member 1
AM1	anthoMATE1
AM3	anthoMATE3
CTAB	Hexadecyltrimethyl-ammonium Bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic acid
$FeCl_3$	Ferric chloride
FW	Fresh Weight
GC-MS	Gas Chromatography - Mass Spectroscopy
GC-TOF-MS	Gas Chromatography - Time Of Flight-Mass Spectroscopy
GLRaV-3	Grapevine Leafrol Virus-3
GSH	Glutathione
HadCM3	Hadley Centre Coupled Model, version 3
MATE	Multi Antimicrobial Extrusion Protein
NAD^+	Nicotinamide Adenine Dinucleotide oxidized
NADH	Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate oxidized
NADPH	Nicotinamide Adenine Dinucleotide Phosphate reduced
OAA	Oxaloacetic acid
OIV	Office International de la Vigne et du Vin
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PVPP	Poly(vinylpolypyrrolidone)
RNA	Ribonucleic acid
SIRT1	Sirtuin 1
SNPs	Single-Nucleotide Polymorphism
SSR	Simple Sequence Repeats
UV-A	Ultraviolet-A
UV-B	Ultraviolet-B

Amino acid notation

Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine	Asn	Methionine	Met
Aspartic acid	Asp	Phenylalanine	Phe
Cysteine	Cys	Proline	Pro
Glutamine	Gln	Serine	Ser
Glutamic acid	Glu	Threonine	Thr
Glycine	Gly	Tryptophan	Trp
Histidine	His	Tyrosine	Tyr
Isoleucine	Ile	Valine	Val

1. Introduction

1 Introduction

1.1 The wine is a "gift from the gods"

The genus *Vitis* includes more than 70 species growing widely in distinct geographical areas (Owens et al., 2008). The most renowned species is *Vitis vinifera* domesticated in Asia Minor or Armenia 5,000 years ago, from where it spreaded to other countries becomig one of the most important horticultural crops cultivated in the world. The high morphological and genetic diversity of *vinifera* has an estimated number of more than 10,000 cultivars (Liang et al., 2011). Worldwide, its cultivated area is approximately 8 Mha. Europe is the most important wine-producing region, and the combined production of Italy, France and Spain nowadays accounts for more than 60% of the world's production of wine. Portugal, Germany, Greece, Russia, Hungary and Romania are also major players in wine production, both in terms of significant vineyard acreage and highly developed viticulture.

Throughout antiquity the conversion of grapes into wine was considered a gift from the gods and the best wines were reserved for the elite of the society. Nowadays, wine is an integral component of the culture of many countries, a form of entertainment in others, and a libation of choice for advocates of its health benefits. Unlike many modern foods, wine's attraction relies not on strong consistent flavors, but upon a subtle array of shifting sensations that make its charm difficult to define (Bisson et al., 2002).

Wine has more than one thousand compounds, that including water, sugars, alcohol, phenolics, acids and mineral salts. The majority of wine compounds, such as vitamins and minerals, come from the grapes, while others, like ethanol and glycerol, are products of the winemaking process (Conde et al., 2007a). This complex composition depends on many and diverse factors, including grape variety, edaphoclimatic conditions and enological practices. The main constituent of wine is, water accounting for 75 to 90% (v/v), and this variation is explained by the amount of the other constituents that form the wine extract that differ from wine to wine. The second largest constituent is ethyl alcohol, which, according to the type of wine, varies from 8% to 15% (v/v). Another important constituent is sugar, which is directly responsible for the final alcoholic content of the wine. A normal dry wine generally has less than 2 g sugar/L, while in a botrytized sweet wine it can reach almost 200 g sugar/L (Dominé et al., 2004).

Most of the wine compounds are produced by the plant itself, in the leaves (including sugars and acids), and in the berry (including acids and phenolics). Furthermore, some molecules related to aroma and tastes are produced during the fruit development and ripening,

being their spectrum specific to a given variety. Theses aromas, called "varietal" or primary aromas, are the grape's signature, recognizable by the consumer during degustation. Thus, the control of growth and the fructification of grapevines in the vineyard are of utmost importance to wine quality (Blouin and Guimberteau, 2000).

1.2 Development and composition of the grape berry

Grape berries comprise three major types of tissue: skin, flesh, and seeds, and exhibit a double sigmoid growth pattern (Coombe, 1992). Growth first occurs mostly by cell division and later by cell expansion (Figure 1). From flowering to approximately 60 days afterward, a first rapid growth phase occurs during which the berry is formed and the seed embryos are produced. Several solutes are accumulated in the berry during the first growth period, contributing in some extent to the expansion of the berry (Possner and Kliewer, 1985).

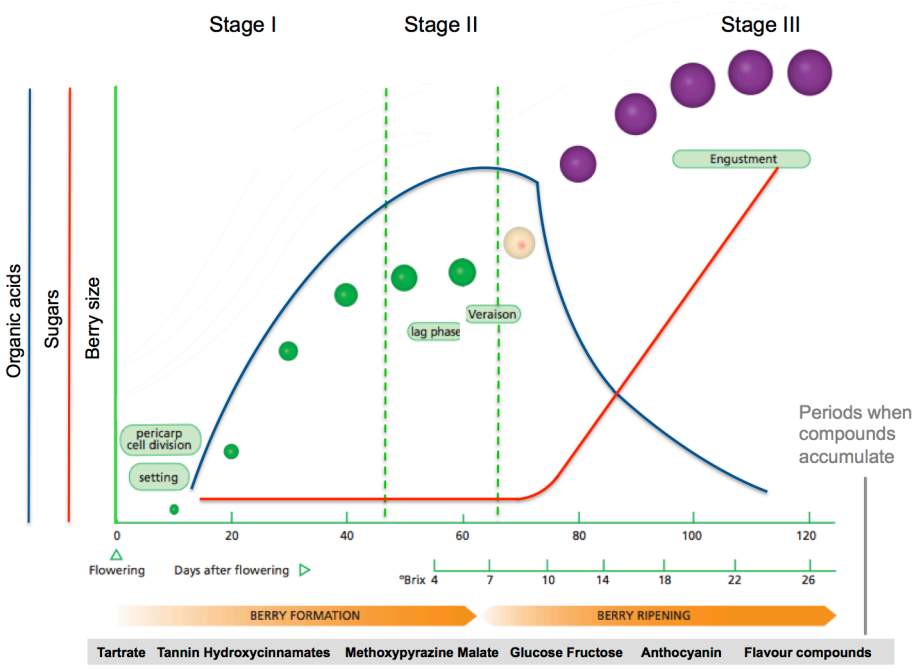


Figure 1. Grape berry development and ripening. Adapted from Kennedy, (2002).

The most prevalent compounds among all the others are by far tartaric and malic acids. Tartaric acid is accumulated during the initial stages of berry development and its concentration is highest at the periphery of the developing berry. By contrast, malic acid is accumulated in the flesh cells at the end of the first growth phase. These acids provide the acidity to the wine, and are therefore critical to its quality (reviewed by Sweetman et al., 2009). Several other compounds like minerals, amino acids, micronutrients, and aroma

compounds also accumulate during the first phase of berry growth and affect as well grape berry quality and ultimately wine quality.

Hydroxycinnamic and hydroxybenzoic acids are also accumulated during the initial growth period. They are distributed in the flesh and skin of the berry and are important for browning reactions and as precursors of volatile phenols (Romeyer et al., 1983).

The French word *veraison* has been adopted to describe the onset of ripening. The most dramatic changes in grape berries composition occurs during this second growth phase, or ripening phase. Berries switch from a status where they are small, hard and acidic, with little sugar, to a status where they are larger, softer, sweeter, less acidic and strongly flavoured and coloured (Figure 2).

Figure 2. Grape berry cluster (cv Padeiro de Basto) at *veraison* stage.

One of the most important ripening-related changes that occur at *veraison* is the beginning of a massive accumulation of sugars. Sugar content and concentration in ripe berries are important parameters for both table grapes and wine. For many decades, particularly in cooler regions, clonal selection and viticultural practices (including row orientation, defoliation and cluster thinning) have been oriented towards high sugar concentrations, which is considered beneficial for wine quality. More recently, an excess of sugars has been observed in many vineyards around the world, which is thought to result from climate change (Davies et al., 2012). The ripening of grape berries is accompanied by a massive accumulation of hexoses that are stored (together with aroma compounds, flavors and ions) in the vacuoles (Fontes et al., 2011). The grape berry is considered to be mainly a sink

for primary metabolites essential for plant survival, and rely on the use of available carbohydrate resources produced by photosynthesis to support growth and development. Sugars transport and allocation between photosynthetic "source tissues" and heterotrophic "sink tissues" is known as assimilate partitioning wich is a major determinant of plant growth and productivity (Kingston-Smith, 2001).

As in the majority of plants, sucrose is the main sugar transported via the phloem in *Vitis*, although several other solutes have been identified, including raffinose, stachiose and the sugar-alcohols mannitol and sorbitol (Conde et al., unpublished). Sucrose derived from leaf photosynthesis is exported via the phloem to the berries. The massive sugar accumulation in berry mesocarp after *veraison* is due to a combined action of monosaccharide (MSTs) and disaccharide transporters (DSTs). These sugar-transport proteins play crucial roles in the cell-to-cell and long-distance distribution of sugars throughout the plant (Figure 3). From *veraison* and throughout ripening the berries accumulate roughly equal amounts of glucose and fructose, reaching over 1 M of each hexose, suggesting that phloem transported sucrose is hydrolyzed at some step during its transport from the leaves to the vacuole of the mesocarp cell (Coombe, 1987; Conde et al., 2006; Conde et al., 2007a; Agasse et al., 2009).

Phenolic compounds such as proanthocyanidins (tannins) and anthocyanins, are responsible for the astringency and colour of wines. These compounds are found mainly in the solid parts of the grapes. From *veraison* to ripening the total content of tannins decreases progressively in the pulp but increases in the seeds and skin (Delgado et al., 2004). Anthocyanins are synthesized from *veraison* onwards, enhancing their concentration in the skin throughout ripening. The flavour that builds in grapes is mostly the result of the acid/sugar balance together with the synthesis of flavour and aromatic compounds or precursors taking place at this period. The development of these characteristics will largely determine the quality of the final product (Boss and Davies, 2001).

Control of the ripening timing, berry size, sugar content and coloration, acidity and the relative assortment of volatile and non-volatile aroma and flavor compounds in table and wine grape cultivars are major concerns to viticulturists, but molecular and biochemical studies on grape berry development and ripening have resulted in significant gains in knowledge (Conde et al., 2007a). In addition, due to their influence in the quality of wine grapes, the effects of environmental factors and viticulture practices on physiological and molecular grapevine responses, including assimilate portioning and secondary metabolism, are also being increasingly under the scope of the scientific community (Castellarin et al., 2007a, 2007b; Vincent et al., 2007; Deluc et al., 2009; Salazar-Parra et al., 2012).

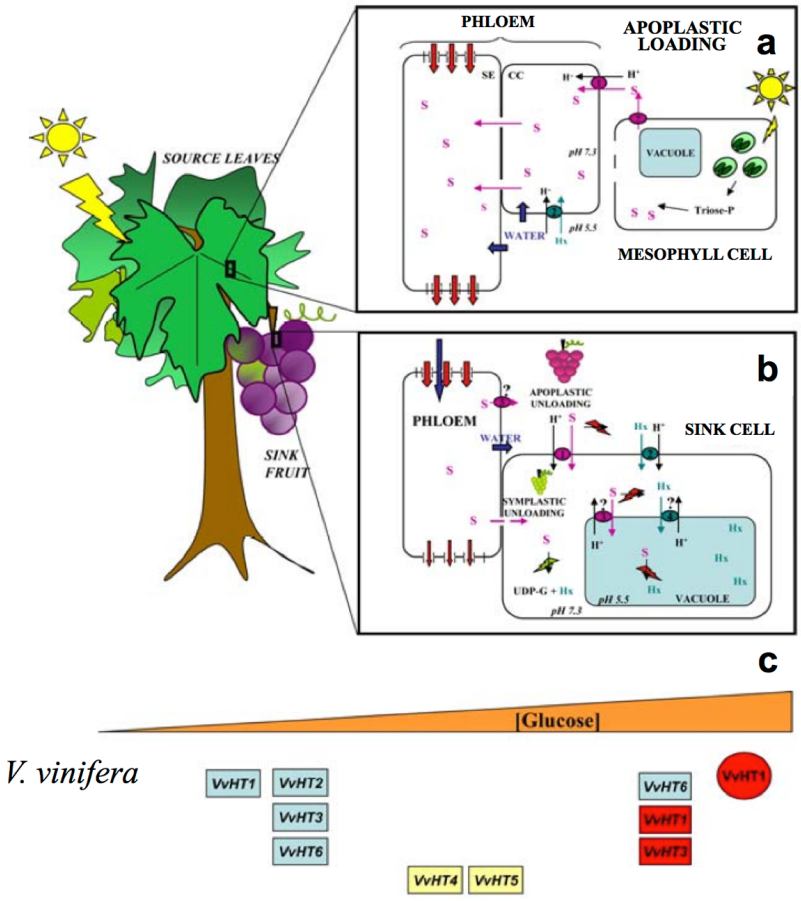


Figure 3. Simplified scheme of long-distance sugar transport in plants. (**a**) Pathways for phloem loading: Sucrose (S) is synthetized in mesophyll cells through photosynthesis. S is loaded into de sieve elements/companion cell complex (SE/CC) via the apoplast. Apoplastic loading involves the retrieval of S leaking from the mesophyll or the vascular parenchyma (mechanism yet uncertain) and may occur along the phloem path. Hydrostatic pressure drives phloem sap movement toward sink tissue. (**b**) Pathways of phloem unloading: S enters the receiving cell by the symplastic route before *veraison*, using plasmodesmata, or the apoplastic pathway after *veraison*. The latter predominates in the ripening fruit and requires the activity of membrane transporters mediating the transport of S, and of the hexoses (Hx) resulting from S hydrolysis by metabolic enzymes (invertases, sucrose synthases). Hx are accumulated in the vacuole. Water fluxes respond to sugar concentration gradient. (1) S/H+ symporter; (2) Hx/H+ symporter; (3) S/H+ antiporter; (4) Hx/H+ antiporter; (5) S efflux transporter; invertase; sucrose synthase; water flux. (**c**) Expression patterns of glucose transporters in *V. vinifera* according to glucose availability. Glucose levels affect both gene (rectangles) expression and protein (circle) amounts. Some transporters are induced (blue) and/or repressed (red) by different levels of glucose or not regulated by sugar concentrations (yellow) (Adapted from Agasse et al. 2009).

1.3 Grape berry composition is affected by environmental conditions and agricultural practices and is cultivar-dependent

Vineyards can be found in Europe, Northern and Southern America, Africa and Asia. In spite of this worldwide distribution, the most important factor for viticulture is climate and, above all, temperature. Grapes clearly prefer moderate conditions, and rarely thrive where temperatures rise above 25ºC in the summer months. In a large part of Western Europe, the location of the majority of European classic viticultural regions, average July temperatures vary between 15 and 25°C. Rainfall and drought also play an important role, and it is almost impossible to grow vines with less than 200 mm of rain a year. A moderate climate, with adequate to relatively high rainfall, provides ideal conditions for producing both fragrant white wines with a good structure and acidity, and well-balanced red wines with good potential for maturing (Dominé, 2004). Wine quality largely depends on the vineyard and on the vine grower.

Grapevine productivity and fruit quality is significantly affected by biotic and abiotic stresses. These perturbations have effects on the synthesis, accumulation and regulation of grapevine numerous compounds. Among several limiting factors that affect growth in Mediterranean-type ecosystems, water deficit, along with high solar radiation and extrime temperatures, are the most important ones. The use of irrigation in these rough environments arises as a solution to avoid excessive canopy temperature, to maintain quality in fruit and wine production and, in more extreme cases, to guarantee plant survival (Chaves et al., 2010). Nevertheless, grapevine irrigation is a subject under considerable debate, as small water supplements may increase yield and maintain or even improve berry quality (Matthews and Anderson, 1989), but on the other hand, may promote excessive vegetative growth with a negative impact on berry color, aromas and sugar content, and in increasing titratable acidity, therefore decreasing wine quality and flavors (Chaves et al., 2010). The large canopy leaf area resulting from prolonged irrigation also tends to increase the incidence of fungal diseases (Dry and Loveys, 1998). Also, water supply is becoming shorter in many regions do to onegoing environmental changes (Deluc et al., 2009).

As described above, grape berries actively accumulate solutes, including sugars, amino acids, sodium and potassium ions and organic acids, decreasing their osmotic potential as response mechanisms to water stress. Also several reports suggest a protective role of secondary metabolites against several stresses, in particular heat, drought and light/UV intensity that severely affect phenolic metabolism and, thus, grape composition and development. The full understanding of how and when specific phenolic compounds

accumulate in the berry, and how the grape berry metabolism of each variety responds to the environment is of utmost importance and is explored in detail in the Section 2.

Besides phenolics (see Section 2), the grape berry content on other metabolites like sugars, organic acids or amino acids also depends on the genotype, environment conditions and agricultural practices.

Some studies suggest that sugar composition of mature grape berry is determined by the genotype (Shiraishi, 2000; Shiraishi, 2010) environment and viticultural practices (Jackson and Lombard, 1993; Kliever and Dokoozlian, 2005). The effects of water deficit on sugar content of grapevine berries are variety-dependent (Gaudillère et al., 2002). Thus, no significant changes were observed in Merlot sugar content under water deficits, while a significant increase in sugar content was observed in Cabernet Sauvignon berries (Castellarin et al., 2007a,b). Similarly, Deluc et al. (2009) observed an increase in berry sugar content under water deficits in Cabernet Sauvignon but not in Chardonnay. This may be explained either by differences in vigour, and therefore source/sink equilibrium, between varieties, or by different mechanisms underlying the response of grape berry development to water limitation according to the timing and intensity of water stress imposition. Indeed, it was shown that water deficit has more effect on berry sugar accumulation when imposed before *veraison* (Keller, 2005; Keller et al., 2006). Moreover, some reports suggest that berry sugar concentration is a relative stable trait for a given cultivar, being less responsive to environmental conditions and viticultural practices than organic acids (Keller, 1998; Sadras, 2007).

More than 20 organic acids have been identified in grape berry with an unusual accumulation of significant concentrations of two organic acids, tartaric and malic, during the berry development and ripening (Ruffner et al., 1982). It as been long known that species within the genus *Vitis* and individual varieties of the cultivated grapevine *Vitis vinifera* show wide variation in the natural acidity of berries. Analyses of acid composition in developing and ripe berries of 26 *Vitis* and 78 varieties of *vinifera* showed differences in levels of tartaric and malic acids (Kliewer et al., 1967). Important research has been dedicated to the effects of heat and light that reaches the berry on acid composition. Lower amounts of malic acid have been generally reported when the berry temperature is increased in relation to respiration stimulation (Ruffner, et al., 1984).

Regarding water stress, in most cases, no titratable acidity changes have been observed in the must from moderately water-stressed vines (Matthews and Anderson, 1989; Esteban et al., 1999). However, some studies report a reduction of titratable acidity in response to deficit irrigation as compared with full irrigation (Sheltie, 2006; Santos et al., 2007). Malate/tartarate

ratio is in general lower due to malate breakdown in vines cultivatd in soils with low water status (Matthews and Anderson, 1989).

Nitrogen-containing compounds found in grapes have also been reported to vary, depending on cultivar, vine nutrition, vineyard management, soil type, soil moisture content, vine virus status, grape maturity and growing season (Kliewer and Lider, 1976; Stines et al., 2000; Bell and Henschke, 2005; Pereira et al., 2006; Conde et al., 2007a). Among amino acids, some studies have shown that proline synthesis is largely increased under severe stress conditions. Either short-term or long-term stress leads to an increment of proline synthesis in plant leaves, but the net photosynthesis remained less affected, and its activity is maintained. Results may suggest that proline synthesis is a survival mechanism of the plant, providing an adaptive potential to acclimate under stress conditions (Sarker et al., 2005).

Like phenolics, some compounds called "varietal" or primary aromas, related to aroma and taste produced during the fruit development and ripening, have a spectrum specific to a given variety (Blouin and Guimberteau, 2000). Some amino acids are precursors to particular volatile compounds and recent studies have demonstrated that the addition of free amino acids increased a number of wine volatiles (Garde-Cerdan and Ancin-Azpilicueta, 2008; Hernandez-Orte et al., 2002).

1.4 Viral infections in grapevine and fruit composition

Viral infections negatively impact grapevine physiology, causing significant economic losses every year (Guidoni et al., 2000; Vega et al., 2011). Grapevine leafroll-associated virus 3 (GLRaV-3), which is responsible for a viral disease at phloem level, belongs to the genus *Ampelovirus* in the family *Closteroviridae* (Martelli et al., 2002; Gouveia et al., 2011), particularly widespread in grapevine population. It occurs in all of the major grape-growing regions of the world, decreasing the strenuousness of affected plants. GLRaV-3 genome consists in a linear monoparticle, positive-sense single-stranded RNA organised into 13 open reading frames (Ling et al., 2004). Grapevine leafroll disease can affect all native and *Vitis vinifera* cultivars, hybrids and rootstocks, infecting only dicotyledonous hosts (Cabaleiro et al., 1997). Even though, symptoms are not expressed in all infected vines (Fuchs, 2007). Infection with GLR virus leads to symptoms such as deformations and discoloration of completely expanded leaves near the end of the growing season (Bovey et al., 1980), and the reduction of yield and quality of grape berries (Goheen, 1988; Beuve et al., 2007). Symptoms are usually most visible in red-fruited cultivars of *V. vinifera* with berries showing pale colouring, due to reduced skin anthocyanin pigments. Leafroll disease leads to 30% to 50%

yield losses (Fuchs, 2007). It depresses berry sugar content delaying fruit ripening and titratable acidity, resulting in reduced wine quality. Some important Portuguese grapevine varieties are presently highly infected, including Cabernet Sauvignon and Touriga Nacional (Gouveia et al., 2011).

The infected plants are more susceptible to adverse environmental factors, such as cold winter temperatures, with subsequent higher level of mortality, forcing more frequent vine replacements and raising the economic losses (Fuchs, 2007). Phenolic compounds are deeply involved in the modifications induced by phloematic viruses in grapevine, with strong implications on the final quality of red wine (Guidoni et al., 2000). Different GLRaV-3 variants are transmitted semi-persistently by coccid or pseudococcid mealybug vectors, according to recent studies (Cabaleiro et al., 1997; Jooste et al., 2011).

Genomic diversity of GLRaV-3 has been scrutinised by several authors in the last years, and they have recognised the existence of divergent numbers of phylogenetic groups, with a range from three to five. However, due to variances in the population of isolates considered and the genes studied, it is still lacking an overview of viral diversity (Jooste et al., 2011; Gouveia et al., 2011). Until now, ten different viruses are known and identified as Grapevine leafroll-associated viruses (GLRaVs), all belonging to the family *Closteroviridae* (Fuchs, 2007). GLR is due to closteroviruses, of which grapevine leafroll-associated closterovirus 1 and 3 (GLRaV-1 and GLRaV-3) are regarded as the most harmful (Guidoni et al., 2000).

As reported above, leafroll has long known effects on grapevine maturity and berry pigmentation. However, the information available is stiel short on physiological and biochemical modifications exerted by the specific viruses on grape berry contente and quality.

1.5 Exploitation of the grapevine genetic diversity to mitigate environmental stress

From the wild species *Vitis sylvestris* spp*,* the ancestor of the cultivated grapevine *Vitis vinifera* spp (Vavilov, 1926; Negrul and Kats, 1946), new diversity of grapevines was developed in few main suitable regions in the Euro-Mediterranean and Central-West Asian territory (Maghradze et al., 2012). The crossing of locally domesticated or introduced from other regions of different grapevine varieties originated thousands of grapevine varieties that have been selected with a wide range of phenotypic traits based on a wide genetic background. Although, as a general consequence of the evolution of viticultural systems and wine making in the last century, as well as the development of national and increasingly globalised international markets, most present day viticulture is based on a very narrow range

of cultivars in comparison to the large genetic diversity that characterized the past viticultural systems.

As discussed in Section 2, the physiology of grapevine has already suffered from significant impacts of global climate change in recent decades causing significant alterations in the biochemistry of the fruit. The combined effect of drought, high air temperature and high evaporative demand during summer in areas like the Mediterranean basin limits grapevine yield and berry development and, consequently, wine quality (Chaves et al., 2007; Costa et al., 2007). To remain productive and competitive, modern viticulture in Europe has to tackle three main concerns: innovation, quality and environmental protection. During the past 30 years, several genetic resources conservation activities have been conducted in the grapevine cultivating countries, mainly through national (public) agricultural research institutions. In this regard, the European COST Action FA1003 – "East-West collaboration for grapevine diversity exploration and mobilization of adaptive traits for breeding" was implemented with the main objective of improving knowledge of the grapevine genetic diversity for its long-term conservation and sustainable use. This Action aimed the characterization of grapevine germplasm, which was accomplished in collaboration with wine growers and professional organizations as key stakeholder groups, to ensure that their demands in terms of characterization and use of traits are taken into account. Thus, scientists and breeders are working together at an international level to generate knowledge about the valuable diversity of grapevine, its patterns, processes and correlations with traits such as resistance and grape quality.

As in other regions of the Mediterranean basin, Portugal has a long tradition in viticulture and a great number of grapevine cultivars. The National List of Grapevine Synonyms contains about 450 varieties. The high number of cultivars in Portugal and their dissemination all over the country resulted in different names being attributed to genetically identical plants (synonymous), although approximately three hundred cultivars are officially recognized. Some of them probably originate from the local wild germplasm, which still has a great diversity of autochthonous grapevine cultivars (Cunha et al., 2010). Nowadays many of them are hardly used and at risk of extinction (Almandanim et al., 2007). Indeed, less than 15 native cultivars represent the majority of those presently utilized for viticulture, namely Alvarinho, Antão Vaz, Arinto, Fernão Pires, for the green yellow cultivars (25 800 ha) and Baga, Castelão, Tinta Barroca, Tinto Cão, Touriga Franca, Touriga Nacional and Trincadeira, for the blue black cultivars (73 630 ha). Others, not of Portuguese origin, like Aragonez (23 500 ha) are also of great importance (Veloso et al., 2010). Traditionally, cultivar characterization relied on plant morphological description (Eiras-Dias et al., 1988). However,

these observations are time consuming and error-prone due to environmental variations that may alter the expression of the measured characteristics.

The development of wine varieties with a strong emphasis on combining wine quality with disease resistance and cold tolerance has been the major target of the grape-breeding programs. Hybridization with resistant cultivars has been one of the few techniques available to develop resistant grape cultivars. However, while improving grape cultivars is possible by conventional breeding, it is difficult and time consuming. New tools of the modern biotechnology such as whole genome sequencing and large scale transcriptomes (microarrays) make the identification of genes involved with valuable traits easier and faster, providing a platform to identify and implement useful genetic engineering strategies for improving biotic/abiotic stress tolerance in grapevines. Taking advantage of the grapevine reference genome it is possible to re-sequence a specific cultivars using low-cost next generation sequencing technologies. This is an important first step to discover large number of genetic markers, generally single nucleotide polymorphisms (SNPs). Recently, a large number of SNPs have been identified in *V. vinifera* varieties and a SNP genotyping array was designed, which will valuable for the assessment of genotype-phenotype relationship (Cabezas et al., 2011).

Over the last years, developments in DNA analysis for the discrimination of cultivars through the application of the microsatellite (SSR) fingerprinting in viticulture have become the technique of choice for cultivar identification and distinction (Bowers et al., 1996; Sefc et al., 1999). According to the OIV, SSR are the best markers to discriminate the cultivars. In fact, This et al. (2004) demonstrated the usefulness of a standard set of microsatellite for identification of grape cultivars.

In order to obtain a Portuguese detailed ampelographic characterization of grapevine germplasm several studies have been performed over the last years with the SSR technique (Lopes et al., 2006; Cunha et al., 2009). Using six nuclear microsatellite loci (VVMD 5, VVMD 7, VVMD 27, VrZAG 62, VrZAG 79 and VVS 2) to study the differentiation of 313 grapevine cultivars officially authorized for wine production in Portugal 244 distinct genotypes were detected and synonyms for 40 cultivars were identified, where 2 to 6 (synonymous) cultivars represent seventeen genotypes (Veloso et al., 2010).

Investment in new varieties that would give good flavors but with improved climate tolerances may be an important investment for the industry and for conservationists wishing to avoid unfavorable land or water use outcomes. Decoupling traditional varieties from regional appellations is an alternative to attempt to maintain varieties. This “managed retreat” to new varieties may reduce water use and upland habitat loss that might be associated with

attempts to retain varieties (Hannah et al., 3013).

After the accomplishing of ampelographic characterization, other studies are needed to identify favorable berry characteristics in response to environmental changing. In this context, projects on large-scale metabolomics of grape berry are providing the basic framework for the characterization of key metabolites and classes of metabolites produced in response to environmental stress.

1.6 Brief characterization of the cultivars used in the present study

Among the white varieties, Alvarinho is considered to be one of the noblest white varieties in Portugal. It was thus chosen to produce the first Portuguese mono-varietal wines and is one of the most used varieties in Vinho Verde production. It is cultivated mostly in North-West of Portugal and produces small and lowly compacted berry clusters. The wines present intense citrus and tropical flavors with fresh acidity. Arinto, also known as Pedernã, is a late ripening variety that produces small berries giving rise to pale wines, aromatic with green tonality and excellent acidity. This grape variety can be used to produce monovarietal citrus colored wines, intensely floral and fruity when young, with high acidity. This variety is highly productive, assuring a perfect balance between acidity, structure, freshness and minerality. Apart from its use in monovarietal wine production, Arinto grapes are also used for blended wines production (Ali et al., 2011). Padeiro de Basto, also known as Tinto Cão, is a highly-productive red variety very frequent in the Vinhos Verdes Demarcated Region. It produces ruby to garnet-red colour wines with a distinctive aroma and taste, harmonious and flavourous. Touriga Nacional is the most important grapevine cultivar in Portugal, with specific characteristics, such as high total acidity and lower pH values at harvest, even in extreme climates (Oliveira et al., 2006). It also has naturally vigorous growth and, subsequently, can grow in low potential soils, such as poor and rocky soils (Guichard et al., 2004). Cabernet Sauvignon is one of the world's most renowned grape variety for production of red wine. It is native of Bordeaux, France, where it grows since the 17th century (Bowers and Meredith, 1997). Characterized by a late and relatively vigorous maturation, with straight new branches, its grapes are highly resistance to bunch rot, especially when grafted in a way that delays the grape maturation (Rizzon and Miele, 2002).

Mature grapes from 21 varieties, five white: Malvasia Fina Perrum, Vital, Antão Vaz, Airén, and sixteen red: Cinsaut, Castelão, Moreto Trincadeira, Jaén, Aragonês Padeiro, Corropio, Tinto Cão, Tinta Miuda, Touriga Nacional, Alfrocheiro, Merlot, Alvarelhão, Alicante Bouschet, and Borraçal, were also used to study phenolics and proline content, and

antiocidant capacity, as described below.

1.7 Objectives of the study

Over the last decade, several metabolomic studies have been performed in grape berries from important *Vitis vinifera* cultivars, including Corvina, Merlot, Touriga Nacional, Alvarinho and Trincadeira (Pereira et al., 2005; Krishnan et al., 2005; Pereira et al., 2006; Son et al., 2009; Ali et al., 2011), but promising research is still underway. In this context, the present work was designed to fulfill two main objectives. The first was to evaluate the influence of edaphoclimatic conditions in the metabolomic profile of grape berries from three Portuguese varieties Alvarinho, Arinto and Padeiro de Basto. To fullfill this task, grapes were sampled in two distinct ampelographic collections, at North (EVAG) and South (INIAV-Dois-Portos) of Portugal, in 2012 season. The second objective was to study genotype-dependent metabolomic profile, when the metabolome of the berry samples from the same region was compared.

In a parallel experiment, mature grape berries from tweenty one selected varieties collected were used to assess the varietal dependence of phenolic content and antioxidant capacity, as well as cultivar dependence of proline content. To accomplish this task, all grape berries were harvested in 2011 season from vines of the ampelographic collection at INIAV (Dois-Portos).

The third objective of the present work was to study how the metabolism of the grape berry is altered in grapevines infected with the Grapevine Leafroll-associated virus 3. Two well-known *V. vinifera* cultivars Cabernet Sauvignon and Touriga Nacional were used in this very preliminary study. Emphasis was given on the effect of virus infection on sugar accumulation and transport in grapes. Also, the activity of the key enzymes mannitol dehydrogenase (MTD) and cinnamate-4-hydroxilase (C4H) was measured in tissue extracts from berries sampled from clean and infected plants. Likewise, the levels of proline, total phenols and anthocyanins were compared in both cultivars in response to virus infection.

2. Berry Phenolics of Grapevine under Challenging Environments

The work presented in this Section has been published:
Teixeira, A; Eiras-Dias, J; Castellarin, S.D; and Gerós, H. Berry Phenolics of Grapevine under Challenging Environments. *Int. J. Mol. Sci.* **2013,** *14*, 18711-18739.

2 Berry phenolics of grapevine under challenging environments

2.1 General characterization of plant phenolics

Plant phenolics have been for many years a theme of major scientific and applied interest. Grape berry phenolics contribute to organoleptic properties, color and protection against environmental challenges. Climate change has already caused significant warming in most grape-growing areas of the world, and the climatic conditions determine, to a large degree, the grape varieties that can be cultivated as well as wine quality. In particular, heat, drought and light/UV intensity severely affect phenolic metabolism and, thus, grape composition and development. In the variety Chardonnay, water stress increases the content of flavonols and decreases the expression of genes involved in biosynthesis of stilbene precursors. Also, polyphenolic profile is greatly dependent on genotype and environmental interactions. This review deals with the diversity and biosynthesis of phenolic compounds in the grape berry, from a general overview to a more detailed level, where the influence of environmental challenges on key phenolic metabolism pathways is approached. The full understanding of how and when specific phenolic compounds accumulate in the berry, and how the varietal grape berry metabolism responds to the environment is of utmost importance to adjust agricultural practices and thus, modify wine profile.

Phenolic compounds can be defined as molecules naturally derived from plants or microbes, consisting of a phenyl ring backbone with a hydroxyl group or other substitutes. Phenolic compounds of the grape are divided between nonflavonoid (with a simple C6 backbone; hydroxybenzoic acids, hydroxycinnamic acids, volatile phenols and stilbenes) and flavonoid compounds (flavones, flavonols, flavanones, flavan-3-ols and anthocyanins). Nonflavonoid phenolics are found in grapes and wine, but with the exception of hydroxycinnamic acids, they are present in low concentrations (Kennedy et al., 2006; Conde et al., 2007a). Flavonoids make up a significant portion of the phenolic material in grapes and include several classes (Conde et al., 2007a). They are C6-C3-C6 polyphenolic compounds, in which two hydroxylated benzene rings, A and B, are joined by a three-carbon chain that is part of a heterocyclic C ring (Figure 4). According to the oxidation state of the C ring, these compounds are divided into structural classes that include flavonols, flavan-3-ols (that include simple flavan-3-ols and their polymeric forms proanthocyanidins), and anthocyanins (Castellarin et al., 2012).

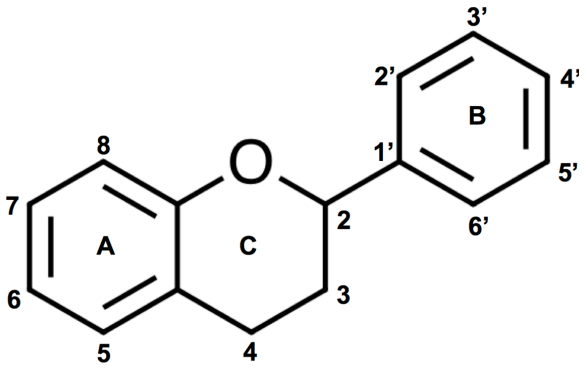


Figure 4. Flavonoid ring structure and numbering.

Grape phenolics contribute to color, flavor, texture and astringency of wine and to its antioxidant properties. The biosynthesis of soluble phenolics begins with the aromatic amino acid phenylalanine, a product of the shikimate pathway. The early precursors of the shikimate pathway are erythrose-4-phosphate and phosphoenol pyruvate. This pathway is responsible for producing phenylalanine and the other amino acids tyrosine and tryptopahne (Conde et al., 2007a; Castellarin et al., 2012).

Although the biosynthesis of many secondary compounds has been elucidated in detail, reports on the identification of transporters of secondary compounds have been published only recently (Braidot et al., 2008; Martinoia et al., 2012) and a clear and precise understanding of flavonoid transport in plants is far from being elucidated.

Two distinguishable tissues compose the grape skin, representing the hydrophobic barrier of the pericarp. The outermost - the epidermis - is strongly cutinized, while the inner thick-walled layers of hypodermis (assumed to consist of several layers, depending on the variety), contain most of the skin flavonoids. In this fraction, the major class of flavonoids is represented by anthocyanins, proanthocyanidins and, to a minor extent, simple flavan-3-ols and flavonols (Braidot et al., 2008). A schematic structure of a ripe grape berry with the distribution pattern of secondary metabolites between tissues is shown in Figure 5.

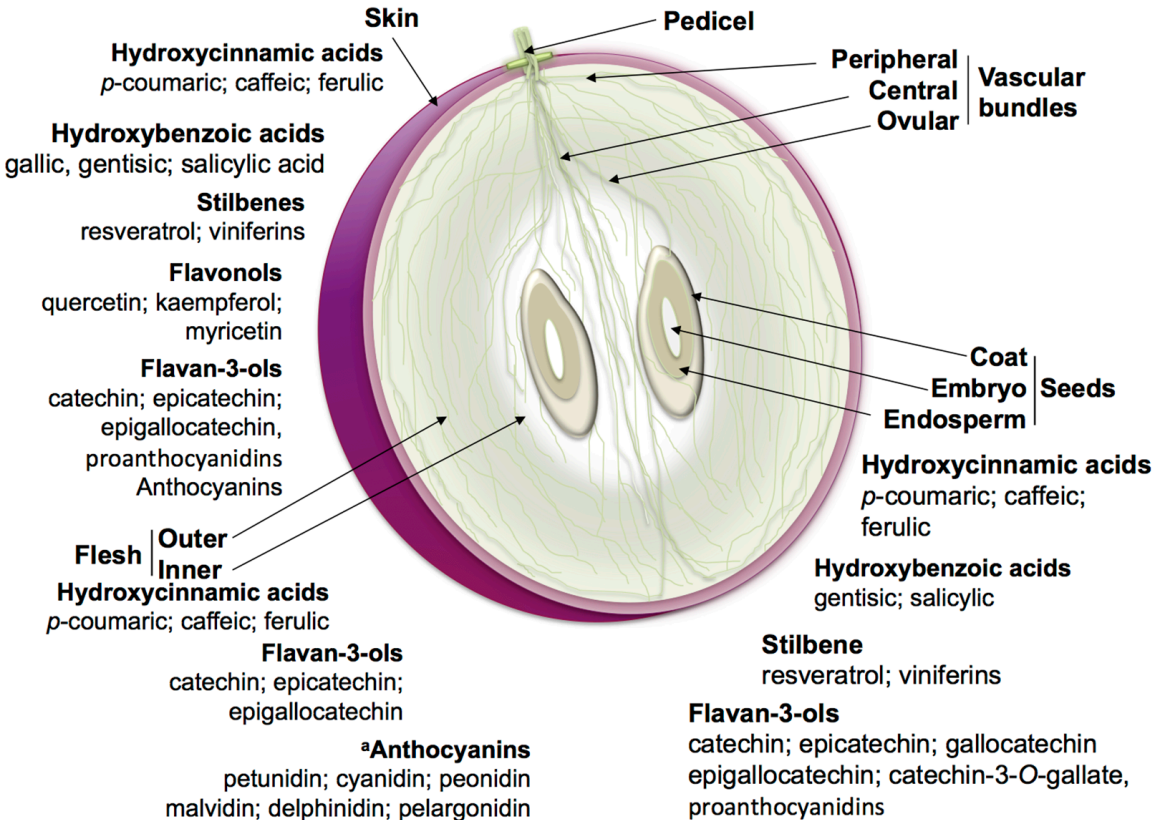


Figure 5. Schematic structure of a ripe grape berry and pattern phenolics biosynthesis distribution between several organs and tissues (indicated by arrows). [a] Anthocyanins are synthetized also in the inner flesh of the teinturier varieties (Coombe, 1987; Bavaresco et al., 1997; Gatto et al., 2008; reviewed by Adams, 2006; Cadot et al., 2006; Conde et al., 2007; Fontes et al., 2011 and Castellarin et al., 2011).

While there is debate about the anthropogenic influence on climate, there are clearly recorded periods of extreme temperature events that may have implications for grape cultivation and wine quality (Easterling, et al., 2000; Chuine et al., 2004; Mann et al., 2009; Cohen et al., 2012) Climate change imposes rapid drifts in weather patterns that determine the suitability of growing regions for specific types of wine (Kenny and Harrison, 1992). Climate changes in the future might extend the north and south latitude boundaries of areas where good wines are produced (Schultz and Jones, 2010). However, some areas that nowadays are producing high quality grapes may be affected by heat and water stress (Kenny and Harrison, 1992). The climate changes are particularly important for grapevine cultivation, in which heat, drought and light intensity are just some environmental stress factors that dramatically affect phenolic metabolism as well as grape development and chemical composition. In this regard, cultural practices, such as canopy management and irrigation may be optimized to adjust berry and wine quality.

Nowadays, the genetic diversity conservation of grapevine is a big concern. The genus *Vitis* contains more than 70 species growing widely in distinct geographical areas (Owens et

al., 2008). The most renowned species is *Vitis vinifera* that was domesticated in Asia Minor or Armenia 5000 years ago, from where it spread to other countries. The high morphological and genetic diversity of vinifera has an estimated number of more than 10,000 cultivars. While many factors, such as viticulture practices, environmental conditions, and post-harvest processing activities, can all affect the content of total polyphenols or individual polyphenolic compounds in grapes and grape products, varietal or genetic difference is one of the most important factors (Liang et al., 2011). The study of the the diversity and biosynthesis of phenolic compounds in the grape berry, from a general approach to a more detailed level, such as the influence of the environmental factors, including drought and heat, and the genotype dependence on the production of grape phenolics, is an important research topic. Also the the comprehension of how and when specific phenolic compounds accumulate in the berry, and how the grape berry metabolism responds to the environment is of utmost importance to adjust agricultural practices and thus, modify wine profile.

2.2 Metabolism and compartmentation of phenolics in the grape berry

Nonflavonoid phenolics

The hydroxycinnamates are the third most abundant class of soluble phenolics in grape berries, after proanthocyanidins and anthocyanins. Phenolic hydroxycinnamates are commonly accumulated in berry skin and the flesh of white and red *vinifera* and *non-vinifera* varieties (Singleton et al., 1986). Thus, while they are also found in red wines, they are usually the most abundant class of phenolics in free-run juice and white wines where they contribute to colour browning under oxidation with non-phenolic molecules (Waterhouse, 2002; Adams, 2006; Kennedy et al., 2006; Conde et al., 2007a). In terms of concentration, *p*-coumaric, caffeic and ferulic acids are also predominant phenolics in grape. These three hydroxycinnamic acids are present primarily as *trans* isomers, although traces of *cis* isomers have been detected. They differ by the type and number of substituents on the aromatic ring. When these hydroxycinnamic acids are esterified with tartaric acid, they are named coutaric acid (*trans*-*p*-coumaroyl-tartaric acid), caftaric acid (*trans*-caffeoyl-tartaric acid), and fertaric acid (*trans*-feruloyl-tartaric acid) (Castellarin et al., 2012).

The synthesis of hydroxycinnamates occurs mainly before *veraison* (Table 1). During ripening, their concentration decreases with the increasing fruit size and dilution of solutes, though its content per berry remains almost constant. Although its accumulation occurs predominantly in the flesh they are present in all berry tissues (Easterling, 2000, reviewed by Braidot et al., 2008) (Figure 5 and Table 1). In hypodermal, mesocarp and placental cells of

the pulp, hydroxycinnamates may be conjugated with anthocyanins (Easterling, 2000; reviewed by Conde et al., 2007a and Castellarin et al., 2012).

The levels of hydroxybenzoic acids and their derivatives are commonly low in wine, compared to the levels of hydroxycinnamic acids. The most common hydroxybenzoic acids in grape berry include gentisic acid, salicylic acid, gallic acid, and *p*-hydroxybenzoic acid, which are mainly found in their free form (Vanhoenacker et al., 2001; Pozo-Bayón et al., 2003; Ali et al., 2010). Gentisic acid is accumulated at very low levels, as is salicylic acid, which is involved in signaling in plants, particularly in the induction of defense and stress responses (Ali et al., 2010; Castellarin et al., 2012). The most represented is gallic acid, which is found free as well as acyl substituent of flavan-3-ols. Other benzoic acids such as protocatechuic, vanillic and syringic acids are found in Riesling wine from Germany (Baderschneider and Winterhalter, 2001). In the seeds, gallic acid can esterify the carbon in position 3 of flavan-3-ols (Adams, 2006).

Table 1. Phenolic compounds produced and accumulated in the grape berry (Langcake et al., 1976; Harbertson, et al., 2002; Downey et al., 2003; Pena-Neira et al., 2004; Adams, 2006; Montealegre et al., 2006; Gatto et al., 2008; Jackson, 2008; Mattivi et al., 2009; Hanlin et al., 2010; Castellarin et al., 2011; Fontes et al., 2011; Castellarin et al., 2012; Martinoia et al., 2012)

Compound	Level of synthesis [a]			Location	Berry phenological scale [b]			
	Skin	Flesh	Seed		Blooming	Green stage	*Veraison*	Ripening
Nonflavonoids								
Hydroxycinnamic acids	++	+++	++	Hypodermal cells and placental cells of the pulp; primarily in the vacuoles of mesocarp cells.	+++	+++	+	+
Hydroxybenzoic acids	+	−	++					
Stilbenes	+++	+	++	Berry skin and seeds.	−	+	++	+++
Flavonoids								
Flavonols	++	−	−	Dermal cell vacuoles of the skin tissue and cell wall of skin and seeds.	++	+	+++	++
Flavan-3-ols	++	+	+++	Specific vacuoles of hypodermal skin cells and seed coat soft parenquima.	+	++	+++	++
Anthocyanins	+++	− *	−	Cell layers below the epidermis; storage confined to the vacuoles and cytoplasmic vesicles named anthocyanoplasts.	−	−	+	+++

a,b Very abundant compound (+++) to absent (−); * Teinturiers contain anthocyanis also in mesocarp cells.

A nonflavonoid compound class that, although present in trace quantities in wine, has been drawing attention is stilbenes (Conde et al., 2007a). These compounds occur naturally in a few edible plants, and several species of the genus *Vitis* are proficient at stilbenes synthesis, mainly in the skin at the mature stage (Figure 5 and Table 1). Stilbene content of the berry changes across varieties (Gatto et al., 2008). Their synthesis also increases upon pathogen infection and in response to abiotic stress (Bavaresco et al., 1997). Some stilbenes, particularly resveratrol, have been drawing attention for their benefits to human health.

Stilbenes can undergo glycosylations or methylations. Glycosylated resveratrol originate piceids, *trans*- and *cis*-resveratrol-3-*O*-β-D-glucopyranosidade as well as astringin, which is a 3′-OH-*trans*-piceid. Modifications by addition of two methyl groups to the resveratrol originate pterostilbene (3,5-dimethoxy-4′-hydroxystilbene) with enhanced antifungal activity compared to the non-methylated form (Chong et al., 2009).

Trans-resveratrol (3,5,4′-trihydroxytilbene) is the stilbene with the simplest molecular structure, which is used as precursor for other compounds through various modifications of the stilbene unit. *Cis*-resveratrol is a *trans*-resveratrol isomer although less stable (Chong et al. 2009). Oligomerisation of stilbenes can be derived in dimers, trimmers and tetramers from oxidative coupling of resveratrol and derivatives by 4-hydroxystilbenes peroxidases. Viniferins are a major group of resveratrol oligomers produced by oxidation of basic stilbenes. The most important viniferins are α- β- γ- δ- ε-viniferins, composed essentially by cyclic oligomers of resveratrol (Castellarin et al., 2012).

Flavonoids

From an anatomical point of view, grape flavonoids are localized mainly in both the peripheral layers of berry pericarp (skin) and in some layers of the seed coat. Most of the skin flavonoids are abundant in the inner thick-walled layers of hypodermis. In this fraction, the major class of flavonoids is represented by anthocyanins, proanthocyanidins (also known as tannins) and, to a minor extent, simple flavan-3-ols and flavonols (Adams, 2006; Braidot et al., 2008) (Figure 5 and Table 1).

Flavonols are a class of flavonoids with a 3-hydroxyflavone backbone. They differ by the number and type of substituents on the B ring (see Introduction), and occur conventionally as glucosides, galactosides, rhamnosides and glucuronides with the sugar bond attached to the 3 position of the flavonoid skeleton. The grape berry synthetizes kaempferol, quercetin, myrcetin and the methylated forms isoharmnetin, laricitrin and syringetin (Mattivi et al., 2006). Flavonols constitute the third component of flavonoids in the skin fraction (Table 1).

Quercetin is known to behave as UV-protectant and to play a role in co-pigmentation with anthocyanins (reviewed by Braidot et al., 2008). As reported below, flavonol concentration varies extensively among varieties, ranging from 0.018 mg to 0.176 mg per g of berry FW, but its content in the berry can be strongly affected by environmental factors, particularly sunlight exposure (among the others, see (Price et al., 1995; Downey et al., 2003; Liang, et al., 2011). Flavonol synthesis occurs primarily during early stages of fruit development and ends at around *veraison* (Downey et al., 2003) (Table 1).

Flavan-3-ols are the most abundant class of phenolics in the grape berry (Singleton, 1992). They have a monomeric (catechins) or polymeric structure known as proantocyanidins or condensed tannins. Catechins and proantocyanidins are located essentially in the seeds, then in the skins and very little in the pulp (Sun et al. 2001). Catechins are responsible for bitterness in wine and may also be partially associated with astringency (Adams, 2006; Kennedy et al., 2006; Conde et al., 2007a). The five flavan-3-ols in grapes are (+)catechin and its isomer (−)epicatechin, (+)gallocatechin, (−)epigallocatechin and catechin-3-*O*-gallate. Catechins are characterized by the presence of a hydroxyl group at the 3 position of the C ring (Su and Singleton, 1969; Waterhouse, 2002; Conde et al., 2007a; Castellarin et al., 2012).

Proantocyanidins are a diverse group of compounds composed by flavan-3-ols polymer subunits that are linked via 4–6 and 4–8 interflavan bonds. These phenolic compounds are the most abundant class of soluble polyphenols in grape berries. Proanthocyanidins vary in size, ranging from dimers to polymers with more than 40 units (Kennedy et al., 2001; Downey et al., 2003; Conde et al., 2007a; Castellarin et al., 2012).

Flavan-3-ols are detectable in highest concentration in seeds (Figure 5 and Table 1). Proanthocyanidins are predominantly found in the hypodermal cell layers of the berry skin and in the soft parenchyma of the seed coat inside the vacuole or bound to cell wall polysaccharides (Adams et al., 2006; Kennedy et al., 2006; Conde et al., 2007a; Castellarin et al., 2012). Grape proanthocyanidins have a larger average size in the skin than in the seeds. These proanthocyanidin compounds are responsible for the grape skin organoleptic properties such as astringency and bitterness in grape skin or wine (Conde et al., 2007a; Braidot et al., 2008).

Anthocyanins are responsible for red, purple and blue pigmentation of the grape berries and, consequently, the red wine. The structures of the common anthocyanins in *V. vinifera* grapes and wine were determined in 1959 (Ribéreau-Gayon, 1959; Conde et al., 2007a). The core of the anthocyanidin, the flavylium, has the typical C6-C3-C6 skeleton. Intrinsically, anthocyanins are glycosides and acylglycosides of anthocyanidins, and the difference of the aglycones and flavyliums (2-phenylbenzopyrilium) occurs at the 3′ and 5′

positions of the B ring, due to hydroxyl or methoxyl substitutions (He et al., 2010a). Anthocyanins can also be esterified by acids, such as acetic, coumaric or caffeic, linked to the 6′ position of the glucose bonded to the 3′ position of the C ring (Conde et al., 2007a; Adams, 2006). There are 17 naturally occurring aglycones, but only six are reported in grapevine: malvidin, cyanidin, peonidin, delphinidin and petunidin. Traces of pelargonidin are found in Pinot Noir and Cabernet Sauvignon (He et al., 2010b), but the malvidin-3-*O*-glucoside was found to be the major anthocyanin present along with its acylated forms (Conde et al., 2007a). *V. vinifera* contains only 3-*O*-monoglycosides due to two mutations in the 5-*O*-glucosyltransferase gene which implicated the loss of the dominant allele involved in the production of diglucosidic anthocyanins (Ford et al., 1998; Jánvár et al., 2009; He et al., 2010a). The anthocyanins commonly found in *V. vinifera* grape include delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides, 3-(6-acetyl)-glucosides and 3-(6-*p*-coumaroyl)-glucosides, peonidin and malvidin 3-(6-caffeoyl)-glucosides, being that malvidin-3-*O*-glucoside is generally the major anthocyanin present along with its acylated forms (Figure 5).

Differently from proanthocyanidin, accumulation of anthocyanin pigments in red grape varieties starts from *veraison* and reaches its maximum in the latest phases of fruit maturation when the synthesis stops (Table 1). Anthocyanins are synthesized in the cytosol of the epidermal cells, are co-localized with proanthocyanidins in the skin hypodermal layers and then stored in the vacuole (Braidot et al., 2008; Fontes et al., 2011) (Figure 5 and Table 1). In a few teinturier varieties, accumulation in the berry skin is paralleled by accumulation in flesh. (Braidot et al., 2008; Castellarin et al., 2012; Falginella et al., 2012). In the red flesh variety Alicante Bouschet, colour development began in the flesh at the stylar end of the fruit and progressed toward the pedicel end flesh and into the skin (Castellarin et al., 2011).

Biosynthesis pathways of phenolic compounds in wine grape

The biosynthetic pathways of different phenolics have been recently thoroughly reviewed by Castellarin et al., (2012) and He et al., (2010a) and are schematically presented in Figure 6.

Hydroxycinnamic acids are generated by modifications to intermediates of the phenylpropanoid pathway. First reaction synthesis of simple phenolics in grape involves the deamination of phenylalanine by the enzyme phenylalanine ammonia lyase (PAL), in which the product is cinnamic acid (Hrazdina et al., 1984). The enzyme cinnamate-4-hydroxylase (C4H) converts cinnamic acid to *p*-coumaric by hydroxylation. *p*-coumaric is esterified by the

enzyme CoA-ligase (4CL) producing 4-coumaroyl-CoA. In these modifications, 3-hydroxylation of *p*-coumaric originate caffeic acid, which can be converted into ferulic acid by 3-methylation. This product is substrate of two enzymes, chalcone synthase (CHS) and stilbene synthase (STS).

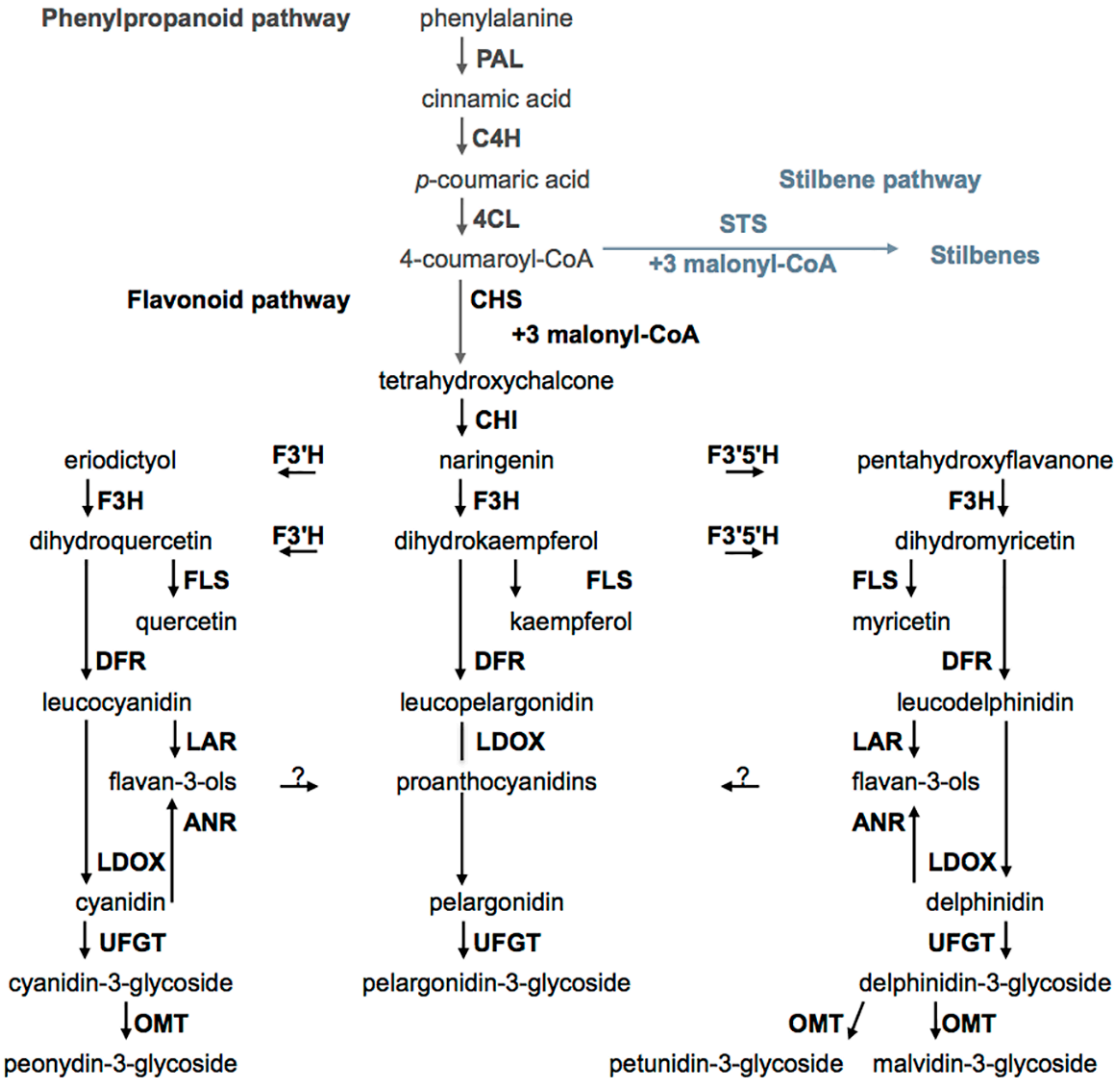


Figure 6. Biosynthetic pathways of grape berry secondary compounds. Phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumaroyl:CoA-ligase (4CL), stilbene synthase (STS), chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3′-hydroxylase (F3’H), flavonoid 3′,5′-hydroxylase (F3′5′H), flavanone-3-hydroxylase (F3H), flavonol synthase (FLS), dihydroflavonol reductase (DFR), leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR), leucoanthocyanidin dioxygenase (LDOX), dihydroflavonol 4-reductase (DFR), flavonoid glucosyltransferase (UFGT), *O*-methyltransferase (OMT). Adapted from He et al. (2010a) and Castellarin et al. (2012).

The first step of the stilbene pathway is controlled by STS. The competition of STS and CHS for the same substrate, 4-coumaroyl-CoA, controls the entry point into the stilbene pathway and flavonoid pathway. In an analogous way of CHS, STS carry out three reactions

of condensation that produce resveratrol. Although, in the STS reaction, the terminal carboxyl group is removed prior to closure of the A ring, causing a different ring-folding in resveratrol compared to the CHS product tetrahydroxychalcone.

All flavonoids stem from tetrahydroxychalcone. The flavonoid pathway leads to the synthesis of different classes of metabolites such as flavonols, flavan-3-ols, proanthocyanidins, and anthocyanins (Figure 6).

Some mechanisms have been proposed concerning flavonoid transport in plants. Flavonoid uptake across the tonoplast may be mediated by a primary active transport, driven by ABC proteins. Very recently it was shown that the ABC protein ABCC1 that localizes to the tonoplast is involved in the transport of glucosylated anthocyanidins, which depends on the presence of GSH but not on the formation of an anthocyanin-GSH conjugate (Francisco et al., 2013). ABCC1 is expressed in the exocarp throughout berry development and ripening, with a significant increase at *veraison*. A genetic screen aimed to study flavonoid biosynthesis provided the first evidence for the involvement of MATE proteins in the transport of flavonoids across the tonoplast. MATE transporters are highly upregulated during maturation, the time when grape berries start to accumulate anthocyanins. It has also been suggested that flavonoid moieties, depending also on their different substituting groups (acyl, glycosyl and/or methoxyl), are driven to their accumulation sites by a complex vesicle trafficking system involving the Golgi apparatus (Braidot et al., 2008). The two grape berry MATEs, anthoMATE1 (AM1) and AM3, specifically transport acylated anthocyanins (Conn et al., 2008; Gomez et al., 2009). Subcellular localization assays revealed that anthoMATE transporters were closely related with these small vesicles, whereas GST was localized in the cytosol around the nucleus, suggesting an association with the endoplasmic reticulum (Gomez et al., 2011). While the biosynthesis and regulation mechanisms of anthocyanin synthesis have been extensively studied, the knowledge on the mechanisms of their sequestration in the vacuole and to what extent their color is affected by vacuole storage is still limited.

2.3 Impact of environment and agricultural practices in grape berry phenolics

Several regional climate models have been proposed in order to forecast the overall effects of individual or combined climate change-related variables (Orduña et al., 2010). Some models take into account air temperature and other variables, including precipitation, humidity, radiation, and historical viticultural records (Stock et al., 2004). Spatial modeling research has indicated potential geographical shifts and/or expansion of viticultural regions with parts of southern Europe becoming too hot to produce high-quality wines and northern

regions becoming viable (Kenny et al., 1992; Schultz et al., 2010; Hannah et al., 2013). For the Northern hemisphere, Jones et al., (2005), predicted that temperatures at regions producing high-quality wine between 2000 and 2049 are going to warmby 0.42 °C per decade and 2.04 °C overall. In the Bordeaux region, the predicted increase temperature overall trend would be 2.3 °C in the same period (Figure 7).

For vineyards, the increase in the number of days with high temperatures is particularly relevant. Grape production and quality are sensitive to heat waves, especially at certain growth stages, such as flowering and ripening. At high temperatures, replacement of starch by lipids in leaf chloroplasts has been reported for grapevines (Buttrose and Hale, 1971). Prolonged periods with temperatures above 30 °C cause a reduction in photosynthesis, with consequent berry size and weight reduction (Hale and Buttrose, 1973). High temperature conditions may have implications in premature *veraison*, berry abscission and reducing flavour development. Metabolic processes and sugar accumulation, beyond other parameters related to colour and aroma, may also be affected or completely stopped by high temperatures (Coombe, 1987; Schultz, 2000; Camps et al., 2012)

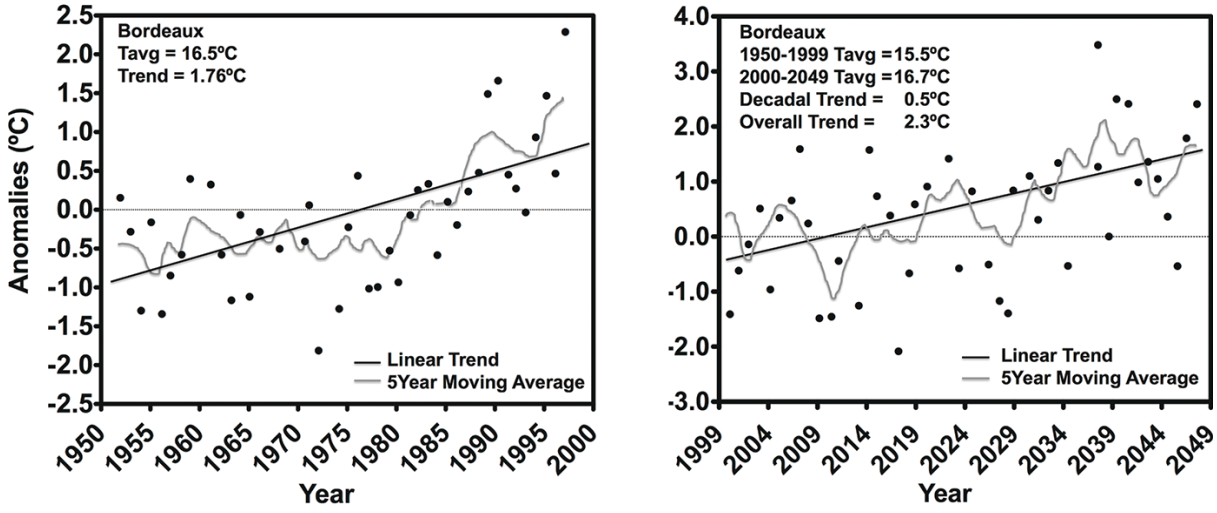


Figure 7. HadCM3 modeled growing season average temperature anomalies for the Bordeaux region. The anomalies are referenced to the 1950 – 1999 base period from the HadCM3 model. Trend values are given as an average decadal change and the total change over the 2000–2049 50-year period. Note: this figure is adapted with permission from Jones et al. (2005). Copyright Springer, 2005.

Studies carried out in European countries have highlighted harvest date advances associated with temperature increases. In southern France, the harvest dates advanced by between 18 and 21 days from 1940 to 2000 (Ganichot, 2002) and in Alsace (eastern France)

the harvest was two weeks earlier in 2002 than in 1972, a period during which temperature increased by 1.8 °C (Duchêne and Schneider, 2005).

In the viticultural French region of Languedoc, the climacteric evolution over the period 1950–2006 obeyed to two distinct climate periods, according to (Laget et al., 2008). Observing the evolution of mean annual and seasonal temperatures, total solar radiation, night freshness index, the distribution and efficiency of rainfall and potential evapotranspiration (pET), it was reported an increase in mean annual temperatures of +1.3 °C between 1980 and 2006 and an increase in the mean pET of 900 mm/year since 1999. It was also concluded that the harvest dates advanced by up to three weeks and sugar concentrations at harvest increased by up to 1.5% potential alcohol. In the Bordeaux region, from 1952 to 1997 changes in the dates of all the phenological events and in the length of the growing season were reported for Cabernet Sauvignon and Merlot (Jones and Davis, 2000). Similar results were found in the southern hemisphere. In Australia, the date of designated maturity of Chardonnay, Cabernet Sauvignon and Shiraz advanced at rates of between 0.5 and 3.1 days per year between 1993 and 2006 (Petrie and Sadras, 2008). A trend towards earlier maturity of several varieties was observed in 12 different Australian winegrape growing regions form 1993–2009 (Webb et al., 2011). For most of the cases, the rate of change in the date of designated maturity was correlated with the rate of change in temperature.

Temperature and radiation

Of environmental factors including all external stimuli, the most influential of which for phenolic synthesis are light/radiation and temperature, as well as water and nutritional status. Phenolic synthesis and accumulation in grape berry is also determined by genetic factors and the interaction between genotype and environment (Orduña, 2010; Castellarin et al., 2012). The role of phenolics as photo-protectants explains their dependency on sun exposure (Orduña, 2010). In warmer climates, high light exposure can increase the concentration of phenolics and anthocyanins because of the higher activity of PAL (Roubelakis-Angelakis and Kliewer, 1986).

Sun exposure is generally considered to be of primary importance for high quality wine production. However, it is not clear whether the effect on fruit composition is due to visible light or ultraviolet light or both (Keller, and Torres-Martinez, 2004).

It has been shown that UV-B provoke several morphological, physiological and biochemical changes in higher plants, depending on the intensity, total dosage, plant species and the balance between UV-B and photosynthetically active radiation (PAR, 400–700 nm)

(Schreiner et al., 2004; Berli et al., 2010). On the other hand, UV-A and visible light may induce both protective and repair mechanisms, thus decreasing the negative impact of UV-B light (Jordan et al., 1992).

However, relatively high levels of solar UV-B were reported to enhance the accumulation of UV-absorbing compounds, including flavonoids and related phenolics (Berli et al., 2008). UV-B is also known to upregulate genes encoding PAL and CHS (Berli et al., 2010). Phenolics transform short-wave, high-energy and highly destructive radiation into longer wavelength light, less destructive to the cellular leaf structures, including the photosynthetic apparatus (Schreiner et al., 2012). Very few studies have attempted to separate the effects of visible light from those of UV light (Schultz, 2000; Kolb et al., 2001). As discussed by Keller (Keller, 2010) this is surprising given that phenolic compounds are absorbed predominantly in the UV range of the spectrum and form an important part of fruit quality in grapes.

Stilbene synthesis is enhanced in response to several abiotic factors. These factors include UV-radiation, wounding, ozone, anoxia and metal ions. Exposure to UV light induces the accumulation of stilbenes in grape berry through the induction of STS expression (Petit et al., 2009). In berries, this is dependent on the development stage, since unripe berries respond to UV irradiation to a greater extent. A study on grape plantlets proved the existence of a positive correlation between resveratrol synthesis in leaves (induced by UV) and field resistance (Sbaghi et al., 1975).

Flavonols are thought to protect plant tissue to UV radiation whereas anthocyanins are thought to provide some protection to UV radiation and high extreme temperatures (Adams, 2006). Synthesis of flavonols is a light-dependent process. Sealing grape bunches in light-excluding boxes from before flowering until harvest completely inhibits flavonol synthesis. If shading is applied later in fruit development, flavonol content is reduced and no further accumulation is detected after the initiation of light deprivation (Price et al., 1995; Spayd, and Tarara, 2002; Downey et al., 2004; Adams, 2006; Castellarin et al., 2012). In Pinot Noir, Shiraz, and Merlot varieties, the amount of these compounds has been shown to be highly dependent on light exposure of the tissues in which they accumulate (Downey et al., 2004). Light modulates the expression of *flavonol synthase* (*VvFLS*), a key flavonol structural gene, and of *VvMYBF1*, a transcriptional regulator of flavonoid synthesis (Czemmel et al., 2009; Azuma et al., 2012; Koyama et al., 2012). In Cabernet Sauvignon and Chardonnay, flavonols are the only phenolic components in both grape leaves and berries that are consistently and severely increased by UV radiation (Keller and Torres-Martinez, 2004). It was suggested that flavonols, but not anthocyanins or hydroxycinnamic acids, are important for UV protection in

grapevine tissues. Similar results were recently confirmed by Koyama et al., (2012) who showed that UV light specifically induced flavonols while not affecting other flavonoid components. However, the relatively high concentrations of flavonols found even in the absence of UV radiation suggest that flavonols may also have a protective function against excess visible radiation (Keller and Torres-Martinez, 2004). In the vineyard, any cultural practices that favor the exposure of grape brunches to sunlight boost flavonol accumulation. This occurs equally in white and red grapes.

Flavan-3-ols and proanthocyanidins are the most stable phenolics under diverse growing conditions. This is also true for accumulation of these compounds in seeds. However, some studies have shown a positive association between temperature and the number of seeds and total proanthocyanidin levels per berry at harvest (Ewart et al., 1977; Del Rio and Kennedy, 2006). Shading treatments increased the amount of seed proanthocyanidins and affected their composition in Pinot Noir (Cortell and Kennedy, 2006) while had no effects in Shiraz (Downey et al., 2004), reiterating the importance to discriminate between irradiation and temperature effects (Orduña, 2010).

Skin flavan-3-ols and proanthocyanidins are more sensitive than seed ones to environmental cues; sunlight has been shown to affect their relative content (Downey et al., 2004; Cortell and Kennedy, 2006; Koyama et al. 2012), as well as their mean degree of polymerization (Cortell and Kennedy, 2006; Koyama et al., 2012). Sunlight exposure consistently increased the relative abundance of the tri-hydroxylated gallocatechins at the expense of the di-hydroxylated catechins and increased the mean degree of polimeryzation.

When the effect of cluster temperature on proanthocyanidins biosynthesis was studied it was shown that there is no consistent relationship between temperature and total proanthocyanidins accumulation across three seasons (Cohen et al., 2012). In this field, experiment grape bunches were cooled during the day and heated at night (±8 °C). However, composition of proanthocyanidins was affected in the experiment because decreasing thermal time in degree-days favored a shift towards tri-hydroxylated forms.

Although anthocyanins and proanthocyanidins share several steps in the biosynthetic pathway, there are many differences in their regulation and reactivity. In fact, in contrast with proanthocyanidins, several authors reported that light, temperature, and their interactive effects, highly influence anthocyanin accumulation in berry skins (Downey et al., 2006; Guidoni et al., 2008). Exposure to sunlight is associated with an increase in anthocyanin accumulation, until the point when excessive heat causes berry temperature to become detrimental (Spayd, and Tarara, 2002; Tarara et al., 2008; Castellarin et al., 2012). In growth chambers, optimal conditions for anthocyanin accumulation occurred when grapes were exposed

to cool nights (15 °C) and mild, temperate days (25 °C) during ripening (Kliewer and Torres, 1972). Higher temperatures (30–35 °C) promote the degradation of the existing anthocyanins (Mori et al., 2007). In the Merlot variety, attenuation of the diurnal temperature fluctuations led to increased ripening rates and higher anthocyanin concentrations at harvest (Cohen et al., 2008). Moreover, absolute anthocyanin levels and chemical composition changes have also been related with warmer seasons, as indicated by the increased formation of malvidin, petunidin, and delphinidin coumaroyl derivatives (Downey et al., 2006). In another study (Tarara et al., 2008), the association of high temperatures with the increase of delphinidin, petunidin and peonidin-based anthocyanins in sun-exposed Merlot berries were observed, while malvidin derivatives remained unaffected. The complexity of combined solar radiation and temperature effects on flavonoid composition further expands the understanding of the effect of such environmental factors on anthocyanin biosynthesis (Orduña, 2010).

Agricultural practices and the levels of synthesized metabolites

In a vineyard, the environment varied due to the natural soil heterogeneity and the uneven light distribution. Physical characteristics of the vineyard can also affect flavonoid accumulation. These include altitude of the cultivation site, heat stress, defoliation, mineral supply or soil type, all of which have shown some influence. Nitrogen, potassium and phosphate are the nutrients commonly applied as fertilizers, although only nitrogen and potassium have thus far attracted viticultural research. Both low and excessively high levels of nitrogen have been shown to decrease color in grape berries, while high potassium has been reported to decrease color in grapes (Delgado et al., 2004; Downey et al., 2006; Kliewer et al., 1977). Despite the age of the soil, which largely determine the micronutrient pool, structure and texture, and significantly affects plant growth (Russell, 1962; Northcote, 1995; Marschner, 1995) the major consequence of soil type is the capacity of the soil to hold water while remaining sufficiently well-drained to avoid waterlogging (Jackson and Lombard, 1993; McDonald et al., 1998; Downey et al., 2006).

Despite the relevance of these parameters, vineyard microclimate has a fundamental influence in the metabolite biosynthesis. The importance of the effect of canopy microclimate on chemical composition of berry was initially raised by Shaulis and co-workers (Shaulis et al., 1966) in their investigations with Concord grapevines. The amount and the distribution of light intercepted by the vines are determined by the architecture of the vineyard, mainly row orientation, height, width, porosity of the canopy, and distance between rows (Pereira et al. 2006). The term “microclimate” was adopted by Smart et al., (1985) to define the

environmental conditions within the immediate vicinity of the leaves and fruit (Haselgrove et al., 2000).

Cultural practice effects on berry have long been studied; among them, leaf removal and cluster thinning, which modify leaf area/yield ratio and fruit-zone microclimate, could potentially improve grape quality (Hunter et al., 1991; Jackson and Lombard, 1993; Dokoozlian and Hirschfelt, 1995; Guidoni et al., 2008). The amount of intercepted light affects the whole plant photosynthetic capacity, water balance, and source to sink balance (Pereira et al., 2006; Castelan-Estrada et al., 2002). The source to sink balance is an important parameter that controls berry sugar, organic acids, and secondary metabolites content with qualitative enological potential (Smart et al., 1990). In general, berries grown under open canopy conditions, compared to berries grown under shaded canopy conditions, have higher juice sugar concentration (measured as total soluble solids), improved acid balance (lower juice pH and higher titratable acidity). However, while some exposure to light may be appropriate, high temperatures resulting from full exposure of berries are likely to inhibit anthocyanin metabolism (Haselgrove et al., 2000).

Vine vigor has been reported to impact upon the proanthocyanidins content and chemical composition of grape skins in Pinot noir. In the berry skin, proanthocyanidins were higher in low-vigor vines, with an increase in the proportion of epi-gallocatechin subunits, as much in polymers as on average size, observed with decreasing vine vigor (Cortell et al., 2005; Downey et al., 2006). It seems that severe canopy shade down regulate gene expression in the anthocyanin biosynthesis pathway, (Jeong et al., 2004; Koyama and Goto-Yamamoto, 2008) while photon fluxes of 100 mmol/m^2/s on the berries temperature becomes the overriding variable in anthocyanin synthesis (Spayd and Tarara, 2002; Downey et al., 2006; Tarara et al., 2008; Keller, 2010).

Among environmental and viticultural parameters investigated in the past decades for various grape varieties, it is known that the water status is a potential modulator of secondary metabolism during the berry development (Hardie and Considine, 1976; Mathews et al., 1987; McCarthy, 1997; Ojeda, 2001). Many scientific articles have extensively reported the effects of water deficit on the accumulation of various grape secondary metabolites (Table 2). Grapevine irrigation can alleviate water-stress-related reductions in plant growth and development, demonstrating the importance of cultural practice at vineyard to guarantee wine quality or even plant survival in regions affected by seasonal drought (Chaves et al., 2007). Several reports demonstrated that large fluxes of water are not essential for the optimal plant performance for agricultural purposes and that moderate water deficits might be used successfully in grapevine production through control of sink-source relationships, thereby

maintaining or ameliorating fruit quality (Chaves et al., 2007). Plant water status affects berry composition, but the effects might be contrasting according to the level and the moment in time when water is applied or deficit is imposed. Furthermore, grape response to moderate irrigation might also be cultivar-dependent as *V. Vinifera* varieties have been shown to respond differently to water stress (Koundouras et al., 2006). Overall, regulation of grapevine water deficit is a powerful tool to manage the amount of secondary metabolite compounds and improve wine quality (Kenned et al., 2002).

The impact of water on stilbene biosynthesis in grapes has been evaluated. The water deficit increases the specific steady state transcript abundance of a STS gene and phenylpropanoid metabolism in general. The increase of STS mRNA abundance suggests an increase in resveratrol accumulation (Grimplet et al., 2007). However, conflicting results have been reported on the effects of water deficit on resveratrol synthesis. Research conducted by Vezzulli et al., (2007) observed little effect of drought on resveratrol concentrations in grape berry skin. In another study on Cabernet Sauvignon and Chardonnay varieties, harvested at six and eight weeks after *veraison*, respectively, Deluc et al., (2011) demonstrated that water deficit increased the accumulation of *trans*-piceid (the glycosylated form of resveratrol) by five-fold in Cabernet Sauvignon berries but not in Chardonnay. However, the abundance of two stilbene-derived compounds - *trans*-piceid and *trans*-resveratrol - was not significantly different between the two cultivars when well-watered. Similarly, water deficit significantly increased the transcript abundance of genes involved in the biosynthesis of stilbene precursors in Cabernet Sauvignon. In contrast, the transcript abundance of the same genes declined in Chardonnay in response to water deficit.

The increased concentration of flavonols, skin-derived proanthocyanidins and anthocyanins has also been observed in wines from grapes grown under the decreased vine water status (Kennedy et al., 2002; Downey et al., 2006).

Recently, it was shown that the concentrations of flavonol increase under drought stress in a white grapevine Chardonnay, but not in a red grapevine Cabernet Sauvignon (Deluc, et al., 2009). Few studies have reported that water deficit may modify the skin proanthocyanidins (Kennedy et al., 2000; Geny et al., 2003; Roby et al., 2004; Chaves et al., 2010) but this topic still awaits further clarification. In Shiraz, the application of water stress before and after *veraison* differently affects the grape berry polyphenol biosynthesis (Ollé et al., 2011). The authors showed that pre-*veraison* water deficit had no effect on total proanthocyanidin accumulation, whereas pre- and post-*veraison* deficits specifically affected the flux of anthocyanin biosynthesis in stressed grape berries sampled with equivalent sugar content. However, both water deficits differently affected the anthocyanin composition. Pre-

veraison water deficit increased anthocyanin accumulation except for malvidin and *p*-coumaroylated derivatives, whereas post-*veraison* water deficit enhanced the overall anthocyanin biosynthesis, particularly malvidin and *p*-coumaroylated derivatives. In Merlot variety under water stress, an increase of anthocyanin content between 37% and 57% for two consecutive years was reported by (Castellarin et al., 2007a).

Imposing water deficits from the onset of ripening until maturity in the Merlot variety reduced the berry weight and increased the concentration of anthocyanins and skin tannins (Bucchetti et al., 2011), and the application of water deficits also modulated chemical composition changes during berry ripening (Castellarin et al., 2007a; Castellarin et al., 2007b).

Table 2. Effect of water deficit on grapevine secondary metabolism.

Variety	Compound	Effect of water deficit	References
Aragonez (Tempranillo)	Anthocyanins	Decreased concentration.	Zarrouk et al., 2012
Barbera	Resveratrol	No effect.	Vezzulli et al., 2007
Cabernet Sauvignon	*Trans*-piceid stilbene precursors	5-fold increase in concentration. Increased transcript abundance of genes involved in the biosynthesis of stilbene precursors and phenylpropanoid metabolism in general.,	McCarthy et al., 1997; Kennedy et al., 2002; Chapman et al., 2005; Downey et al., 2006; Castellarin et al., 2007b; Grimplet, et al., 2007; Deluc et al., 2009; Deluc et al., 2011
	Flavonols	Increased concentration in the skin and in the wine. No changes in seeds.	
	Anthocyanins	Increased of concentration in the skin and in the wine. Increased expression of many genes responsible for their biosynthesis.	
Chardonnay	Stilbene precursors	Increased concentration.	Deluc et al., 2009
	Flavonols	Decreased transcript abundance of biosynthetic genes.	
Merlot	Anthocyanins	Increased concentration and biosynthesis;	Castellarin et al., 2007a; Bucchetti et al., 2011
	Proanthocyanidins	Increased concentration in berry skin.	
Shiraz	Anthocyanins	Increased concentration.	Ollé et al., 2011

When Aragonez (Syn. Tempranillo) grapevines were subjected to three irrigation regimes (conventional sustained deficit irrigation (DI), regulated deficit irrigation (RDI) and non-irrigated (NI), the main compounds affected by water availability were proanthocyanidins and flavonols which were increased with irrigation at pea size, veraison, mid-ripening and full maturation phenological stages (Zarrouk et al., 2012). Concentrations of anthocyanin at full maturation were observed to be higher in the skin of berries belonging to DI and RDI vines than in NI ones. In general, although no differences in sugar accumulation were observed between the water treatments, a decrease in the quality parameters in grape skins in NI vines was observed, may resulting from high temperature and excessive cluster sunlight exposition.

2.4 Varietal dependence on grape berry phenolics

Traditionally, morphological and agronomical characteristics have been the main criteria for differentiating grapevine cultivars, but it is well known that many of those characters are strongly influenced by environmental conditions (Pomar et al., 2005). Grapevine varieties are not genetically homogeneous and intravarietal diversity varies across cultivars (Moncada et al. 2005; Stajner et al. 2009). Even vines multiplied by vegetative propagation display a broad range of characteristics (Anderson et al., 2008). As referred to in the introduction, the grape phenolic profile depends greatly on the grape variety (Gatto, et al., 2008; Mattivi et al., 2006; Yang et al., 2009; Katalinić et al., 2010). In a recent study, (Liang et al., 2011) showed that the polyphenol profile revealed significant differences among 344 European grape varieties. Polyphenol variations among several varieties are summarized in Table 3.

Table 3. Varietal differences in the grape berry composition.

Varietiy	Nonflavonoids			Flavonoids			References
	Hydroxycinnamic acids mg·g^{-1} FW	Hydroxybenzoic acids mg·g^{-1} FW	Stilbenes mg·g^{-1} FW	Flavonols mg·g^{-1} FW	Flavan-3-ols mg·g^{-1} FW	Anthocyanins mg·g^{-1} FW	
Araclinos	0.742	0.034	0.001	0.042	0.386	0.655	Liang et al., 2011
Aragonez						0.658	Arozarena et al., 2002
Cabernet Sauvignon	0.103	0.011	0.003-0.095	0.039	1.830	1.830 - 1.084	Arozarena et al., 2002; Bavaresco et al., 1997; Castillo-Muñoz et al., 2007
Chardonnay	0.138	0.022			0.129		Liang et al., 2011
Coudsi	0.088	0.008	0.012	0.018	0.128		Liang et al., 2011
Garnacha						0.474	Castillo-Muñoz et al., 2007
Greco di Tufo			0.0002				Gatto et al., 2008
Melon	0.822			0.049			Liang et al., 2011
Pinot Noir	0.152	0.018	0.003	0.035	0.161	0.800	Gatto et al., 2008; Liang et al., 2011
Rofar Vidor	0.402	0.081		0.053	0.440	0.655	
Royalty			0.002	0.148	0.734	5.123	Liang et al., 2011
Sauvignon Blanc	0.221	0.035	0.003	0.022	0.123		
Touriga Nacional	0.754	0.024	0.006	0.176	0.33	2.632	

Phenolics from grape and wine have generated remarkable interest with their antioxidant and free radical scavenging properties. Catechins, proanthocyanidins and anthocyanins are the most concentrated natural antioxidants present in red grape and wine (Conde et al., 2007a; Mattivi et al. 2002) and it is believed that they play important beneficial roles in the mammalian systems (Lafay et al., 2009). The differences in phenolic composition observed across varieties might impact their respective health benefits.

Owing to its biological and agricultural importance, the genetics and biochemistry of the flavonoid biosynthetic pathway have been widely studied and the great intravarietal variability recommends the use of more precise methods to characterize and classify grape germplasm collections. Methods used to track back the variety and for producing a given wine rely on the composition in proteins, amino acids and aroma compounds, or on DNA analysis (Siret et al., 2000; Hernández-Orte et al., 2001; Pomar et al., 2005). To a certain extent, flavonol profiles have demonstrated that some of them can be used as chemical markers for the authentication and varietal differentiation of grapes and wines (Garde-Cerdán et al., 2009). Among those metabolic compounds, which have frequently been used as chemical markers in chemotaxonomy, in recent years the cultivar-characteristic profiles of monomeric anthocyanins have been widely used for the classification and differentiation of grape cultivars and monovarietal wines (Singleton, 1992; Monagas et al., 2003; Fanzone et al., 2011). Despite the strong role of the genetic background in determining the composition of anthocyanins, the content of anthocyanins in grapes changes during their maturation and seasonal conditions, and the physical and chemical characteristics of the soil also influence the distribution of anthocyanins in grapes (Arozarena et al., 2002; Pomar et al., 2005). For example, Downey et al., (2004) found that the anthocyanin fingerprint was altered by cluster exposition or shading to sunlight, by temperature regimes reached during the growing season, and by water deficit treatments (Castellarin et al., 2007a). Moreover, Guidoni et al., (2003) stated that cluster thinning changed the proportion of anthocyanins, increasing cyanidin and peonidin 3-*O*-glucosides whereas malvidin 3-*O*-glucoside and acylated anthocyanins were not affected. The relative proportion of anthocyanins also varies during grape ripening; however, this composition is practically constant in the final stages of ripening (Ryan et al., 2003). Nevertheless, most references coincide with the fact that the non-genetic factors such as several environmental conditions or viticultural practices have a greater effect on the concentration of anthocyanins rather than on their relative composition (Arozarena et al., 2002; Pomar et al., 2005). Moreover, it is commonly accepted that anthocyanin concentration of grape berry also varies according to the genetic background, which is independent of seasonal conditions or production area (Ortega-Regules et al., 2006).

2.5 Conclusions and future perspectives

Grapevine phenolics play distinctive roles during the development of the fruit until full maturation. Hydroxybenzoic acids may be involved in signaling, particularly in the induction of defense and stress responses, and stilbenes are effective antifungal agents. Flavonols are thought to act as UV and extreme temperature protectants, as well as free radical scavengers. The astringency role of proanthocyanidins (condensed tannins) is thought to act as a feeding deterrent to herbivorous and other insects. Anthocyanins play important roles in DNA protection and defense against photo-oxidative stress. In wine, hydroxycinnamates contribute to colour browning under oxidation in association with molecules. Also, proanthocyanidins contribute to mouthfell of red wine, as well as colour stability by forming complexes with anthocyanins that are responsible for the colour, and also contribute to the sensory attributes of wine. Important nutraceutical and pharmacologic properties have also been attributed to grape berry phenolics, including antimicrobial, anticarcinogenic and antioxidant. Several reports indicate that *trans*-resveratrol inhibits the proliferation of tumor cells and had a putative protection against diabetes. Their role against neurodegenerative diseases were recently postulated due to the resveratrol ability to activate the protein SIRT1 that was related to many diseases associated with aging (Hubbard et al., 2013). Thus, the continued study of grape phenolics has an important basic and applied relevance.

The physiology of grapevine has already suffered from significant impacts of global climate change in recent decades. Harvest occurs sooner and sooner, although grape growers tend to wait longer for ripeness. Berry sugar content (and alcohol in the wine) tends to increase whereas phenolic and aromatic ripeness are not always achieved. Acidity tends to decrease with potential effects on wine aging capacity. Water supply is becoming shorter in many regions (Delrot et al., 2010). The site and season conditions are the most important factors that influence phenolic content of a grape cultivar. In particular, light and temperature affect to a great extent the phenolic content of the berry. These parameters are the most difficult to manage, although some viticulture practices, including strategic use of irrigation, utilization of cover crops, row orientation, trellising, and other canopy modifications may optimize plant interaction with light and temperature. Thus, the development of management strategies for optimizing grapevine phenolic composition in challenging environments is an important issue in modern viticulture. The improvement and implementation of standardized

tools to quantitatively and qualitatively measure flavonoids in the grape berry is also an important research topic that could provide important developments in the future.

Although the inherent plasticity of grapevine response to environmental conditions may account for phenolic variation, several evidences introduced in this review show that phenolic profile is very dependent on the genotype. In this regard, the selection of new varieties with pleasant sensorial flavors but with improved climate tolerance may be an important investment for viticulturists and the wine industry. To address this challenge, scientists and breeders need to work together at an international level to generate knowledge about the valuable diversity, and patterns, processes and correlations with traits such as resistance and grape quality, which is the aim of the ongoing European Cost Action COSTFA1003 "East-West Collaboration for Grapevine Diversity Exploration and Mobilization of Adaptive Traits for Breeding" (2010–2013). For instance, despite the large number of studies on grape colour, there is still not a complete understanding of the genetics underlying this phenotype. In this regard, specific genes significantly associated with total skin and pulp anthocyanin were recently detected in red and rose cultivars from the Portuguese Ampelographic Collection, suggesting their involvement in anthocyanin content (Cardoso et al., 2012).

Important efforts have been undertaken by several research laboratories worldwide to understand and enhance the mechanisms of phenolic biosynthesis in grapevine, but this area of basic research is still widely open. Although the biosynthesis of many secondary compounds was already elucidated in some plants, the identification and characterization of specific transport steps have been published only recently, but a complete understanding of flavonoid transport and compartmentation in grape berry tissues in response to the environment is far from being elucidated. In addition, how the networks of phenolic biosynthesis are regulated and coordinated in different varieties, tissues and environments remains to be uncovered. In this regard, future investigation will involve the exploration of grapevine genetic diversity and the study of the role of specific genes or metabolic pathways in response to environmental conditions, taking advantage of the already available grapevine reference genome.

3. Material and Methods

3 Material and Methods

3.1 Berry sampling

Grape berries from three wine varieties – Alvarinho, Arinto and Padeiro de Basto -, were collected in 2012 season in two different Portuguese ampelographic collections located in North - Demarcated Region of Vinho Verde (Estação Vitivinícola Amândio Galhano - EVAG) and South – Estremadura Region (Instituto Nacional de Investigação Agrária e Veternária - INIAV). From each variety three clusters from tree different vines were collected at different phenological stages: green pea, *veraison* and mature at 18 ºBrix. Each sample was stored separately and carried in a thermal luggage. Grape berries were ground with mortar and pestle in liquid nitrogen. The powder was stored at -80 ºC for posterior use.

Grape berries from Cabernet Souvignon and Touriga Nacional clean and infected with the Grapevine Leaf Roll virus (GLRa-V3) were also collected in 2012 season in the ampelographic collection of Instituto Nacional de Investigação Agrária e Veternária (INIAV). Three clusters from each clean and infected variety at the mature stage were sampled. Each sample was stored separately and carried in a thermal luggage. Part of the samples were ground with mortar and pestle in liquid nitrogen. The powder was stored at -80 ºC for posterior use.

In a different approach, grape berries from twenty one grape varieties, five white (Malvasia Fina Perrum, Vital, Antão Vaz, Airén) and sixteen red (Cinsaut, Castelão, Moreto Trincadeira, Jaén, Aragonês Padeiro, Corropio, Tinto Cão, Tinta Miuda, Touriga Nacional, Alfrocheiro, Merlot, Alvarelhão, Alicante Bouschet, and Borraçal) were collected in 2011 season in the ampelographic collection of the Instituto Nacional de Investigação Agrária e Veternária (INIAV) to study in berry tissues genotype-dependent phenolics and proline content and antioxidant capacity. For each variety, three clusters at the mature stage were collected from three different vines. Each sample was stored separately and carried in a thermal luggage. The samples were ground with mortar and pestle in liquid nitrogen. The powder was stored at -80 ºC for posterior use.

3.2 Sample preparation for metabolomic analysis

To provide detailed information on the grape berry metabolome in vines from two different regions the above-mentioned powder was lyophilized for six days. Metabolite extraction from the lyophilized samples and analysis by GC-TOF-MS were carried out at UC

Davis Genome Center Metabolomics Laboratory, as described by Fiehn et al. (2008). After metabolite extraction and derivatization, samples were injected in split-less mode with a cold injection system (Gerstel, Germany) and analyzed by GC (Agilent 6890, San Jose, USA) using a Rtx 5Sil MS column (30 m x 0.25 mm, 0.25 µm film thickness) and an integrated guard column (Restek, Bellefonte, USA). The GC was connected to a Leco Pegasus IV TOFMS spectrometer controlled with Leco ChromaTOF software v.2.32 (Leco, St. Joseph, USA). Peak detection and mass spectra deconvolution were performed with Leco Chroma-TOF software v.2.25. GC-MS chromatograms were processed as described by Fiehn et al. (2008). Further analysis after deconvolution was made using the semi-automated workflow of the UC Davis Genome Center Metabolomics Laboratory (Fiehn et al., 2005). Metabolite data were normalized using the dry weight (DW) of the lyophilized samples. For all experimental conditions, three independent runs were performed in all metabolomic analysis.

3.3 Quantification of free amino acids

Whole grape berries at mature stage from North and South regions were ground in liquid nitrogen and lyophilized for six days. Extraction was performed by adding 25 ml of milli-Q H_2O to 1 g of grape berry powder and quantification of natural free amino acids (excluding tryptophan) was performed in a Biochrom 30 Aminoacid Analyser with a weak acidic cation exchange resin acting as stationary phase (200 x 4.6 mm column) and a number of weak acidic Li-citrate buffers acting as mobile phase. Stepwise pH, temperature and salt concentration gradients were applied. Detection after post column derivatization with Ninhydrin (135°C) at 570 or 440 nm was performed (Ansynth Service, B.V.). For tryptophan quantification, the sample solutions were diluted in milli-Q H2O (1:10) and analysis was performed using a Beckman System Gold HPLC equipped with an Allsphere C8, 250 x 4.6 mm (stationary phase) and using a phosphate buffer/MeOH gradient (mobile phase). Detection was performed by fluorimetry, with emission wavelength set at 340 nm and excitation wavelength set at 280 nm (Ansynth Service, B.V.).

3.4 Enzyme extraction from berry tissues

Extraction of total protein from frozen powders was performed as described by Stoop and Pharr (1993) with some alterations. Each powder was mixed with the extraction buffer in an approximately 1:1 (v/v) powder:buffer ratio. The protein extraction buffer contained 100 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT) and 1% (v/v)

Triton X-100. The homogenates were then centrifuged at 18,000 *xg* for 20 min at 4 ºC and the supernatants were kept on ice. The total protein content was determined spectrophotometrically by the Bradford method (Bradford, 1976), with bovine serum albumin (BSA) as the standard.

3.5 Enzymatic activities

Malate dehydrogenase activity

The extracts for MDH activity were obtained as described above. MDH activity assay was performed at 30ºC as described by Taureilles-Saurel (1995a) with some modifications. The reaction medium contained 50 mM of Trisaminometano (Tris-HCl) (pH 8), 1.7 mM $NaHCO_3$, 1.3 mM $MgSO_4$, 1.7 EDTA, 0.3 mM NADH and 3 mM of oxaloacetic acid in a final volume of 1 mL. MDH activity was determined spectrophotometrically at 340 nm by measuring the rate of NADH-dependent reduction of oxaloacetate to malate. The reaction was started by the addition of 0.05 mL of extract after equilibrium.

$NADP^+$-dependent malic enzyme activity

The extracts for $NADP^+$ malic enzyme activity were obtained as described above. $NADP^+$ malic enzyme activity assay was performed at 30 ºC, in a total volume of 1 ml. The reaction mixture contained enzyme extract, 100 mM Tris-HCl (pH 7.4), 0.3 mM $NADP^+$, and 30 mM of malic acid (pH 7.4) to ensure the V_{max} of the enzyme (different concentrations of malic acid were used for kinetics studies). The reduction of $NADP^+$ was evaluated spectrophotometrically at 334 nm using a double beam spectrophotometer. One of the cells contained the reaction mixture and the blank was placed in the other. All reactions were initiated by the addition of 0.05 mL of protein extract.

Mannitol dehydrogenase (MTD) activity

The extracts for MTD activity were obtained as described above in the presence of 1% (w/w) of PVPP. MTD activity was assayed at 37 ºC, in a total volume of 1 ml. The reaction mixture contained enzyme extract, 300 mM Bis-Tris propane (pH 9.0), 1mM NAD^+, and 200 mM of D-mannitol to ensure the V_{max} of the enzyme (Conde et al, 2007b). MTD activity was determined by measuring the rate of mannitol-dependent conversion of NAD^+ to NADH spectrophotometrically at 340 nm. All reactions were initiated by the addition of mannitol.

Cinnamate 4-hydroxylase (C4H) activity

The extracts for C4H activity were obtained as described above in the presence of 1% (w/w) of PVPP. C4H activity was assayed at 37 ºC as described by Jian-Ye et al. (2006) with some modifications. The reaction mixture contained enzyme extract, 300 mM Bis-Tris propane (pH 8.9), 150 μM NADPH, 1.25 mM glucose 6-Pi, and 125 μM of *trans*-cinnamic acid (different concentrations of *trans*-cinnamic acid were used for kinetics studies) in a total volume of 1 ml. C4H activity was determined spectrophotometrically at 340 nm by measuring the rate of NADPH-dependent conversion of *trans*-cinnamic acid to *p*-coumaric acid. All reactions were initiated by the addition of *trans*-cinnamic acid.

3.6 Determination of proline content

Proline was extracted from 200 mg of frozen powder in 1.8 mL (w/v) of sulfosalycilic acid. The homogenate was centrifuged at 18,000 *xg* for 20 min^{-1} and 1 mL of supernatant was added to 2 mL of a solution containing 1 mL of glacial acetic acid plus 1 mL of acid ninhydrin solution (0.025 g of ninhydrin, 0.4 mL phosphoric acid and 0.6 mL of acetic acid). After 1h incubation in a boiling water bath (100ºC), the reaction was stopped on ice. Afterwards 2 mL of toluene was added and mixed vigorously. The separation of the aqueous from the organic phase allowed the extraction of the proline chromophore from the organic upper phase (Wren et al., 1965; Bates et al., 1973). Free proline was evaluated spectrophotometrically at 520 nm and its concentration was quantified using a proline standard (Sigma-Aldrich, St. Louis, USA).

3.7 Total Phenolic content

Determination of total phenolics was performed by Folin-Ciocalteau colorimetry method (Waterhouse, 2002). Total phenolics from 100 mg of grape berry powder were extracted in 1 mL of methanol (100 %). The homogenates were shacked for 15 min. and then centrifuged at 18,000 *xg* for 20 min. Twenty μL of supernatant were added to 1.58 mL of deionized water plus 100 μL of folin reagent, vigorously shacked an incubated for 5 min. in the dark before adding 300 μL of sodium carbonate (2M). After 2 h of incubation in the dark the absorbance of the samples were measured at 765 nm. The phenolic concentrations were determined using a gallic acid [GAE] calibration curve.

3.8 Antioxidant profile of twenty-one Portuguese grapevine varieties

The determination of the reducing potential of de grape berry extracts followed the method FRAP (Ferric Reducing Antioxidant Power, Benzie & Strain, 1996). In this assay, antioxidant capacity was evaluated by the reduction of Fe^{3+} to Fe^{2+}, which is chelated by 2,4,6-tripyridil-S-triazine (TPTZ) to form a Fe^{2+}–TPTZ complex absorbing at 593 nm. Total phenolics from 100 mg grape berry powder were extracted in 1 mL of methanol (100 %) and 10 μL of the extract was added to 0.6 mL of working solution previously warmed at 37 ºC containing 2.5 mL of acetate buffer (0.3 M, pH 3.6), 0.25 mL of TPTZ solution (10 mM) and 0.25 mL $FeCl_3$ (20 mM). Water was added to a final volume of 1 mL. The reaction mixture was maintained at 37 ºC for 15 min. and the absorbance of the samples was measured at 593 nm. All solutions were used fresh. The antioxidant efficiency of grape extracts were determined using a Fe^{2+}calibration curve.

3.9 Sugar content in berries from vines clean and infected vines with Grapevine Leafrol Virus-3

The extracts were obtained by adding 1 mL of water : ethanol (1:5) to 100 mg of frozen powder and vigorously shacked. The homogenates were collected in a 2 mL eppendorf tube, boiled for 10 min. and centrifuged for 5 min. at 15,000 *xg*. The supernatant was collected and the solvent evaporated under a N_2 flow. Ultrapure water (500 µL) was added to the extract before sonication for 10 min. (Breia, 2011). The extract was filtered with PTFE 0.2 μm filter before injection.

Chromatographic analyses were carried out on a Hitachi Auto sampler L-2200 Elite LaChrom chromatograph coupled to an RI detector. The injections were 20 μL and the flow rate was kept constant throughout the analysis at 0.5 $mL.min^{-1}$ at 60 ºC. The column used was a Rezex RCM monosaccharide Ca^{+2} (8%) and the eluent was water. Sugar concentrations of each sample were determined by comparison of the pick area with established calibration curves of each compound.

3.10 Expression of sugar transporters in berries from clean and infected vines with Grapevine Leafrol Virus

RNA extraction procedure

The RNA extraction was done whith the *RNeay Plant Mini Kit* (QIAGEN) except that the extraction buffer was different. Briefly, 100 mg of powder was mixed with 1 mL of extraction buffer (2% CTAB, 2% PUP, 100 mM Tris HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% β-mercaptoetanol) and incubated at 60ºC for 20 min^{-1} whith occasional vortexing. The remaining procedures followed the instructions of the manufacturer. The integrity of the RNA was evaluated in agarose gels stained with SYBR® Safe (Invitrogen™, Life Technologies) and quantified using Quantity One® Software (Bio-Rad Laboratories, Inc.).

cDNA synthesis

The cDNA synthesis was performed whith the *Omniscript RT Kit* (QIAGEN). The reverse transcriptase reaction medium containing 2 μL of buffer, 2 μL of oligo dT, 2 μL of dNTPs, 2.5 μL of RNase inhibitor, 1 μL of reverse transcriptase, 1 μg of RNA and 10.5 μL of RNase free water in a final volume of 20 μL. The reaction was performed at 37ºC for 1h.

Expression studies of VvHT1 and VvHT6 by RT-PCR

VvHT1 and *VvHT6* primers were designed to amplify the coding region of each gene (Supplementary files). For *VvHT1*, the forward and reverse primers were, respectively, 5'-CAC GTC CAT GGC TCC GTT CTT GCA GAA GTT C-3' (with 31 bp, Tm = 65.7ºC, cytosine-guanine = 54.8%), and 5'- GCC ATC TCA GAG AGG TAG AGC GGC ACA GA-3' (29 bp, Tm = 65.7ºC; cytosine-guanine = 58.6%. The *VvHT6* forward and reverse primers were, respectively, 5'-CAT ATC GGA TTG GAT TGG TCG GC-3' (23 bp, Tm = 57.1ºC, cytosine-guanine = 52.2%) and 5'- TCT TCA GTA AGC TCA CCA GTT GG CC-3'(25 bp, Tm = 59.3ºC, cytosine-guanine = 52.0%).

The RT-PCR reaction was performed with the kit *Hot Star Taq* (QIAGEN). The reaction mixture contained 10 µL Hotstar Taq master mix, 1 µL of primer forward, 1 µL of primer reverse, 1 μL of cDNA, and 7 μL DNase free water to a final volume of 20 µL. Polymerase was activated with an initial step of 15 min at 95°C to enzyme activation, the double strand denaturation occurred at 94ºC, the annealing temperature was 55 ºC and the

extension temperature was 72ºC. The amplification of *VvHT1 and VvHT6* was achieved after 40 cycles for both varieties.

The forward and reverse actin primers used as controls were respectively 5'- GTG CCT GCC ATG TAT GTT GCC ATT CAG GCT G-3' and 5'GCT CTT TGC AGT TTC CAG CTC TTG CTC GTA GTC A-3' amplified for 28 cycles. RT–PCR products were separated in agarose gels, stained with SYBR® Safe (Invitrogen™, Life Technologies) and quantified using Quantity One® Software (Bio-Rad Laboratories, Inc.).

3.11 Total anthocyanin content in berries from clean and infected vines with Grapevine Leafrol Virus

The total anthocyanins were quantified by a pH differential method. The extraction of total anthocyanins was performed by adding 1 mL of acetone (100%) to 200 mg DW. After mixing on a shaker for 10 min. the mixture was centrifuged at 18,000 *xg* for 20 min. and sonicated for 10 min^{-1}. Two dilutions of the same sample were prepared in 25 mM KCl (pH 1.0) and sodium acetate 25 mM (pH 4.5). The quantification of anthocyanin content was evaluated spectrophotometrically at 520 nm and 700 nm. The total anthocyanin concentration was obtained from the equation:

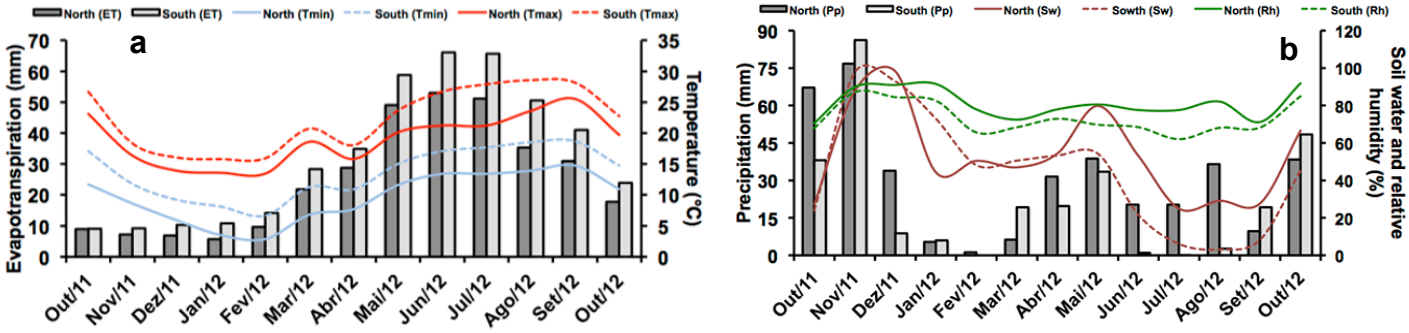
$$[\text{total anthocyanins}](\text{mg/L}) = \frac{\Delta A \times MW \times 1000 \times DF}{\varepsilon}$$, where $\Delta\mathbf{A}$ is the absorbance variation between wavelengths, MW is the anthocyanin molecular weight, DF is the dilution factor and $\boldsymbol{\varepsilon}$ is the molecular absorbance coefficient (Nicoué et al., 2007).

4. Results

4 Results

4.1 Climateric characterization of the sampling regions

Figure 8 shows the meterological and agrometeorological elements from two regions very close to the vineyeards where grape samples were collected (EVAG and INIAV-Dois Portos, respectively): Viana do Castelo (North) and Lisboa (South). This figure refers to the time period of October 2011 to October 2012 and was constructed from the data provided by Instituto Português do Mar e da Atmosfera (IPMA, http://www.ipma.pt/pt/; ISSN 0870-4694). As can be seen, North and South regions were very different in what regards climatological characteristics. In the South the air temperature and the evapotranspiration were consistently higher than in the North during all the time period. In addition, the higher amount of precipitation in the North region resulted in higher relative humidity and soil water content than in the South region (Figure 8a and b).



a)
Evapotranspiration north (ET) - dark grey bars;
Evapotranspiration south (ET) - light grey bars south;
T_{min} North - hatched blue line;
T_{min} South - continuous blue line;
T_{max} North - hatched red line;
T_{max} South - continuous red line.

b)
Precipitation north (Pp) - dark grey bars;
Precipitation south (Pp) - light grey bars;
Soil water content north (Sw) - continuous brown line;
Soil water content south (Sw) - hatched brown line;
Relative humidity north (Rh) - continuous green line;
Relative humidity south (Rh) - hatched green line.

Figure 8. Meteorological and agrometeorological elements from North (Viana do Castelo; close to EVAG) and South (Lisboa; close to INIA - Dois Portos) regions from October 2011 until October 2012 (http://www.ipma.pt/pt/; ISSN 0870-4694).

4.2 Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal

GC-TOF-MS broad analysis

As referred to in Material and Methods, for this study three grape varieties were selected – Alvarinho, Arinto and Padeiro de Basto -, and grape berries were sampled in 2012 season in two different ampelographic collections located in the North of Portugal (EVAG) and in the South (INIAV). Grape berries were harvested at green pea, *veraison* and mature stages. When grape berries reached the technological stage of 18 ºBrix it was conventioned that the mature stage was achieved. A total of 84 metabolites were detected by GC-TOF-MS analysis ranging from sugars, organic acids, amino acids and polyols to some quite unexpected solutes, like urea (Supplementary Table 1).

Several sugars were detected in grape berries from all varieties and sampling places including sucrose, glucose, fructose, rhamnose, levanbiose and inulotriose (Supplementary Table 1). As expected, glucose, fructose and, in minor amounts, sucrose were the most important sugars. Also, equivalent levels of glucose and fructose were always detected for each sample. In general, the pattern of sugar accumulation did not change between varieties, from *veraison* to mature stage. In addition, given that grape berries were sampled at the same brix, the total amount of sugar at the mature stage did not change significantly. The most noticeable differences were observed in the levels of sucrose at the mature stage in Alvarinho and Padeiro de Basto cultivated in the North and South. Indeed, as shown in Figure 9d and f, the amounts of sucrose are much lower in grapes from the South region. In Alvarinho and Arinto cultivars, this reduction in sucrose amount coincides with slightly higher levels of glucose and fructose, suggesting that in the South the invertase activity is stimulated.

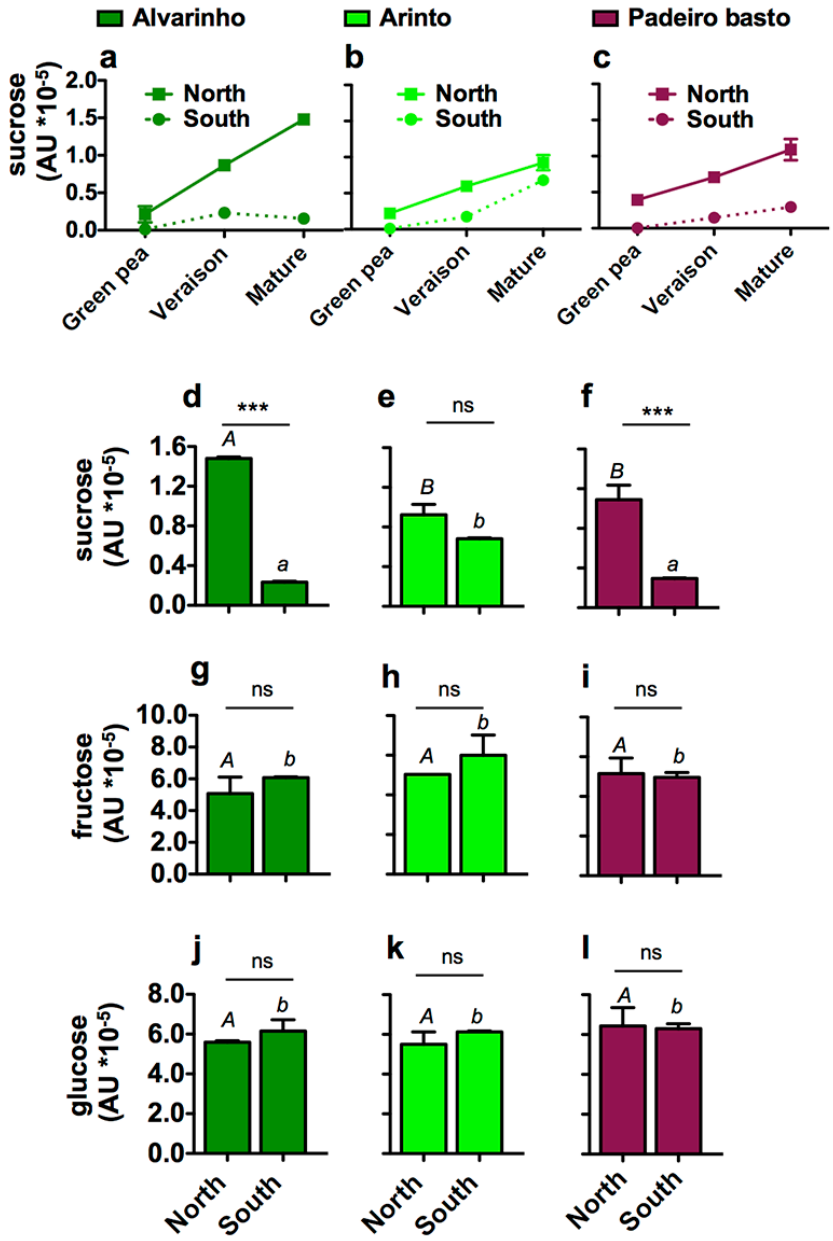


Figure 9. Qualitative metabolome analysis (sugars) of grape berries from Alvarinho, Arinto and Padeiro de Basto cultivated in the North and South Portugal. Sucrose levels during grape berry development and ripening (**a-c**). Sucrose, fructose, and glucose levels at the mature stage (**d-i**). Values are the mean ± SEM (n=3). Asterisks above the bars indicate one-way ANOVA statistical significance (*** $P < 0.001$; ns = non significant) between the same varieties in different regions; letters above the bars indicate one-way ANOVA statistical significance ($P< 0.05$) between grapes from different varieties cultivated in the same region (upper case: north varieties, lower case: south varieties).

Besides tartaric and malic acids – the most abundant organic acids in the berry -, the present metabolome analysis also detected in all samples minor amounts of other organic acids, including citric, fumaric, and succinic (Figure 10 and Supplementary Table 1). Noticeably, maleic acid was very abundant in all samples and in much higher levels than its

trans-isomer, the fumaric acid. Maleic acid and fumaric acid do not spontaneously interconvert because the rotation around a carbon-carbon double bond is energetically unfavourable.

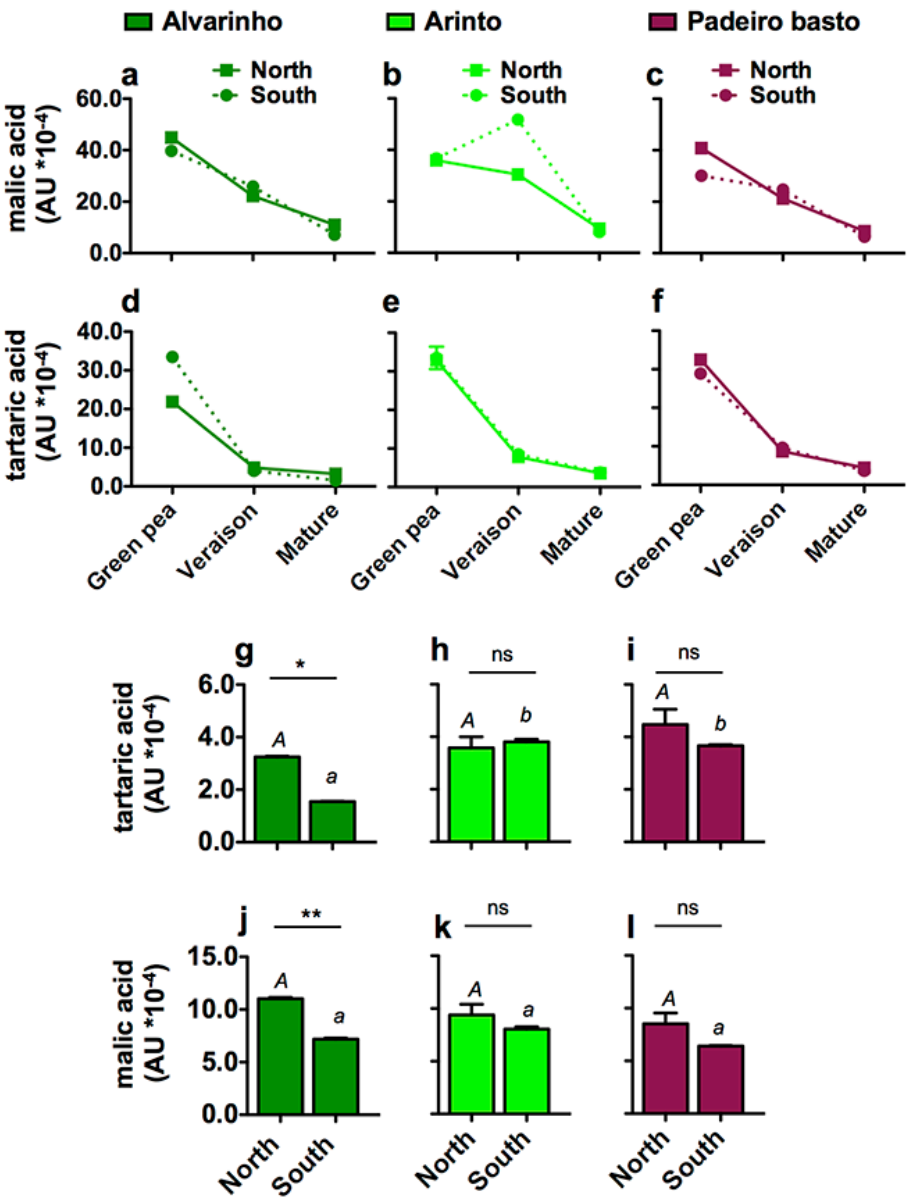


Figure 10. Qualitative metabolome analyses (organic acids) of grape berries from Alvarinho, Arinto and Padeiro de Basto cultivated in the North and South Portugal. Malic acid levels (**a-c**) and tartaric acid levels (**d-f**) during grape berry development and ripening. Tartaric acid and malic acid levels at the mature stage (**g-i**). Values are the mean ± SEM (n=3). Asterisks above the bars indicate one-way ANOVA statistical significance (* P < 0.05; **P < 0.01; ns = non significant) between the same varieties in different regions; letters above the bars indicate oneway ANOVA statistical significance (P< 0.05) between grapes from different varieties cultivated in the same region (upper case: north varieties, lower case: south varieties).

The amount of organic acids decreased during grape berry development and ripening and, as observed for sugars, the pattern of organic acid variation was similar between varieties, as shown in Figure 10 for tartaric acid and malic acid. The grape berries from the variety Alvarinho cultivated in the North showed higher levels of malic acid and lower levels of tartaric acid than the other two varieties, although the differences were not significant (Figure 10d and g). The sampling place seemed to affect the levels of tartaric and malic acid in the grape berries. The most important statistically significant difference was observed in mature grapes from Alvarinho that contained 52% and 35% less tartaric and malic acid, respectively, in Dois Portos than in EVAG vineyards.

In a recent study Conde et al. unpublished) described that *Vitis vinifera* Mannitol Transporter 1 (VvPLT1) has a physiological role in drought tolerance as it is overexpressed in grape berry pulp tissues at the latter ripening stages, thus contributing to increased unloading rates and significant accumulation of polyols in the fruit under severe water stress conditions. In the present metabolome analysis sorbitol, ribitol, galactinol erythritol were also detected in all varieties (Supplementary Table 1).

Sorbitol seemed to be the most important polyol in grape berries from Padeiro de Basto and its levels steadily increased after *veraison* contrarily to the observed in the other two varieties. At the mature stage the levels of sorbitol in Padeiro de Basto where 7.3-fold and 13-fold higher than in Alvarinho and Arinto, respectively, in the North region (Figure 11d-f). In the varieties cultivated in the South the levels of polyols were generally higher than in the North, although the differences were only statistically significant in the case of galactinol in grape berries from Arinto. Yet, in Alvarinho the amounts of ribitol in the berries were lower in the South than in the North.

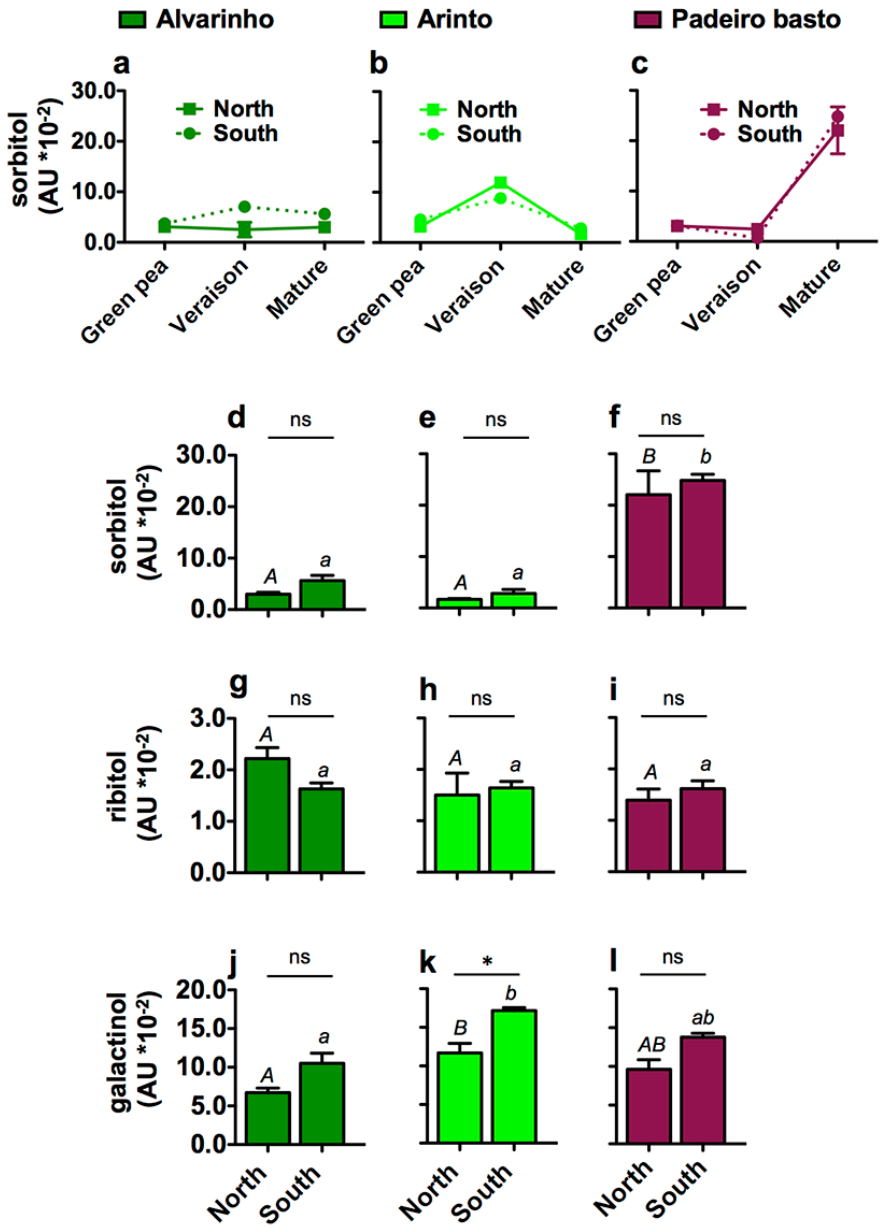


Figure 11. Qualitative metabolome analysis (polyols) of grape berries from Alvarinho, Arinto and Padeiro de Basto cultivated in the North and South Portugal. Sorbitol levels during grape berry development and ripening (**a-c**). Sorbitol, ribitol, and galactinol levels at the mature stage (**d-i**). Values are the mean ± SEM (n=3). Asterisks above the bars indicate one-way ANOVA statistical significance (* $P < 0.05$; ns = non significant) between the same varieties in different region; letters above the bars indicate one-way ANOVA statistical significance ($P< 0.05$) between grapes from different varieties cultivated in the same region (upper case: north varieties, lower case: south varieties).

As explored in the Section 2, grape berry phenolics contribute to organoleptic properties, colour and protection against environmental challenges. In the present metabolome analysis only few secondary metabolites were detected, including well-known hydroxycinnamates, such as benzoic acid, 3,4-dihydroxibenzoic and caffeic acid, catechin and

epicatechin (Figures 12-13 and Supplementary Table 1). The pattern of nonflavonoid accumulation/degradation during development and ripening of the grape berries was dependent on the cultivar and on the collecting region, in particular for benzoic acid, as shown in Figure 12. At the mature stage the levels of benzoic acid were significantly affected by the edaphoclimatic conditions in Alvarinho and Arinto cultivars because benzoic acid levels were 5.7-fold and 3.7-fold higher, respectively, in the South region than in the North. Contrarily, the levels of caffeic acid seemed to be slightly lower in grape berries from the varieties cultivated in the South, but the differences were not statistically significant.

Catechin levels during the development and ripening of the grape berries followed a similar decreasing pattern for all varieties cultivated in Dois Portos or EVAG. At maturity, catechins are more abundant in the white varieties Alvarinho and Arinto than in Padeiro de Basto. The catechin isomer, epicatechin, is more abundant in grape berries from Arinto than in Alvarinho and Padeiro de Basto. The growing region affected the levels of catechin and epicatechin in grape berries from all varieties. Catechins decreased in average by 30% from the North to the South while epicatechins decreased by 48% (Figure 13).

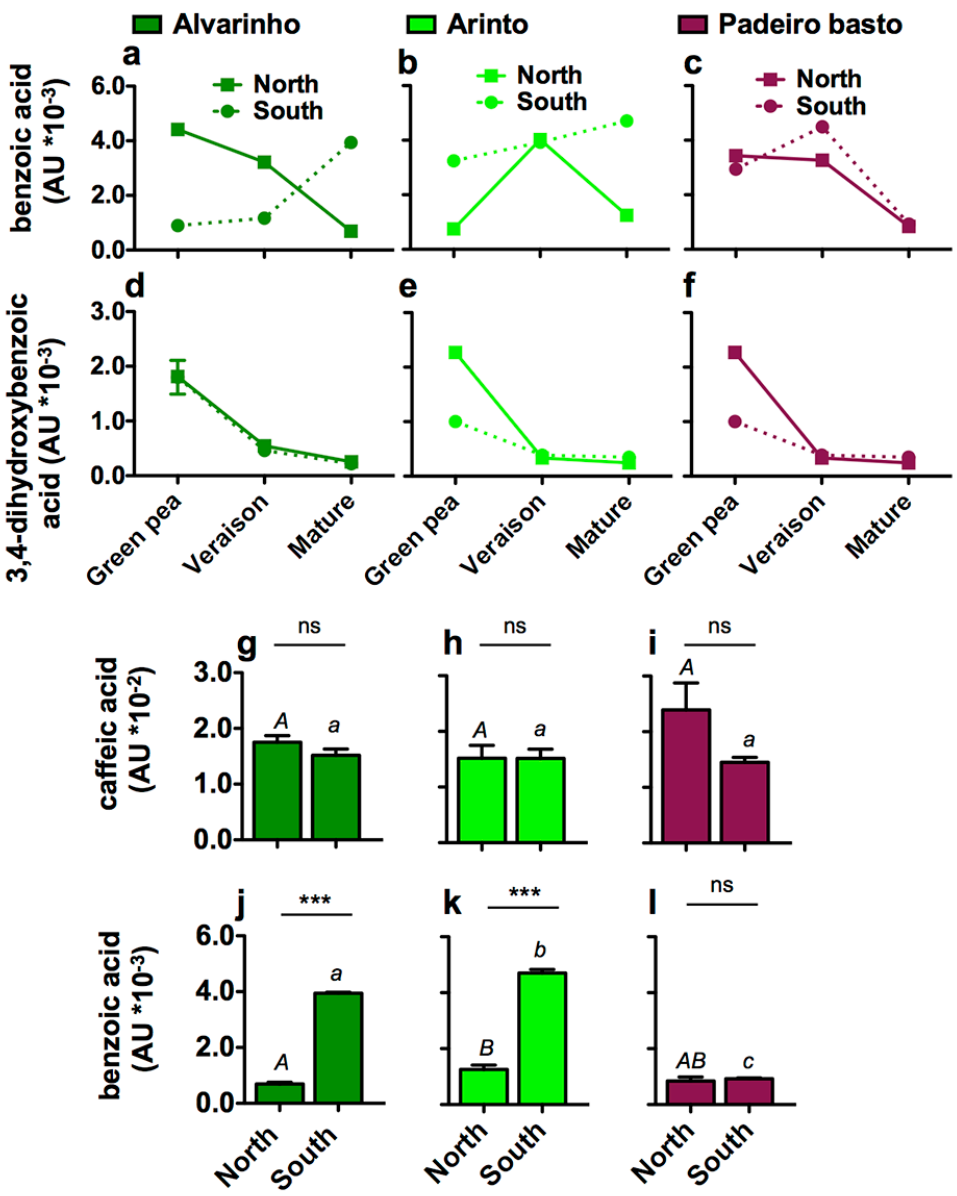


Figure 12. Qualitative metabolome analysis (non-flavonoid phenolics) of grape berries from Alvarinho, Arinto and Padeiro de Basto cultivated in the North and South Portugal. Benzoic acid and dihydroxybenzoic acid levels during grape berry development and ripening (**a-f**). Caffeic acid and benzoic acid levels at the mature stage (**g-l**). Values are the mean ± SEM (n=3). Asterisks above the bars indicate one-way ANOVA statistical significance (* $P < 0.05$; ***$P < 0.001$; ns = non significant) between the same varieties in different region; letters above the bars indicate oneway ANOVA statistical significance ($P< 0.05$) between grapes from different varieties cultivated in the same region (upper case: north varieties, lower case: south varieties).

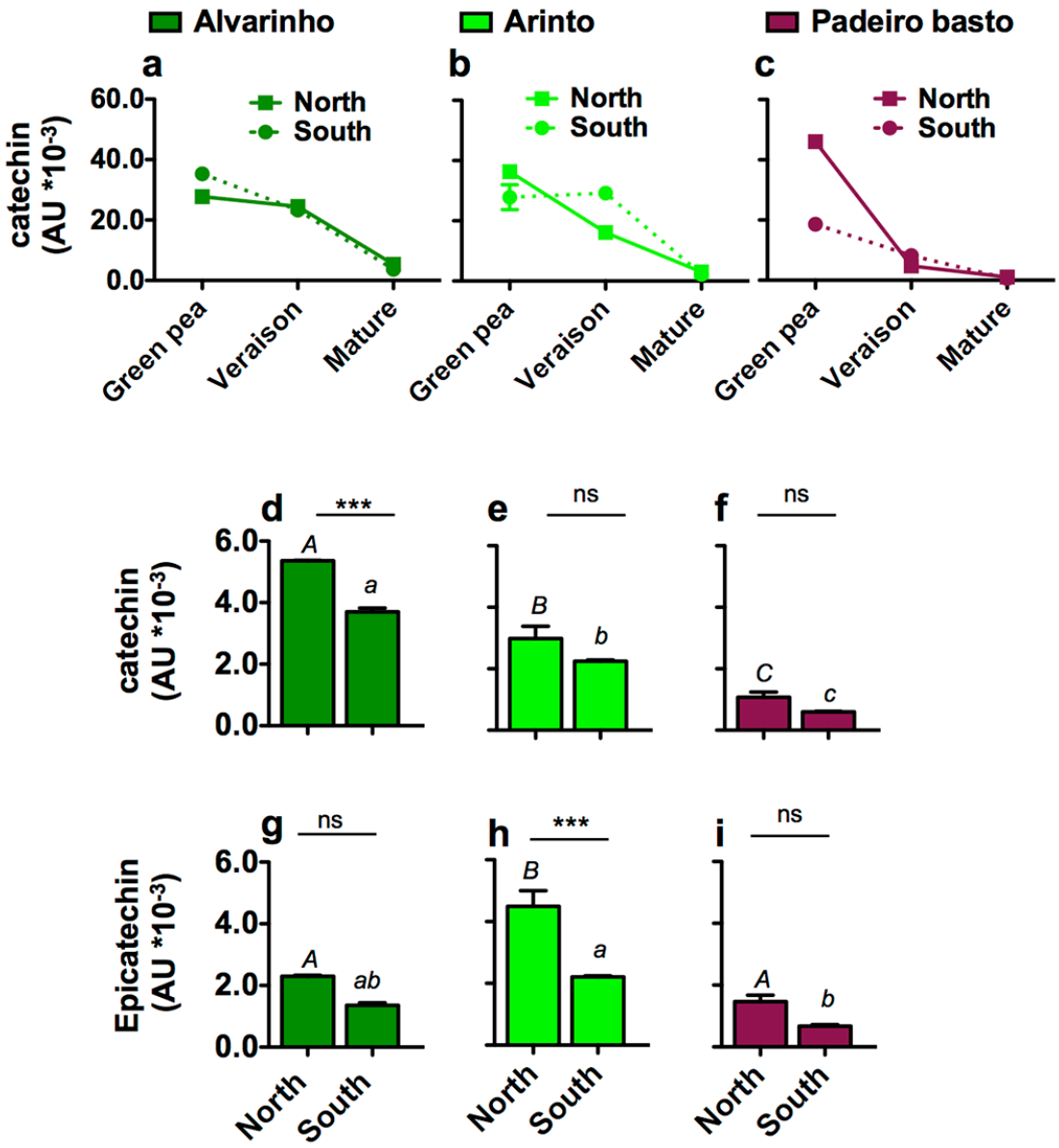


Figure 13. Qualitative metabolome analysis (flavan-3-ols) of grape berries from Alvarinho, Arinto and Padeiro de Basto cultivated in the North and South Portugal. Catechin levels during grape berry development and ripening (**a-c**). Catechin and epicatechin levels at the mature stage (**d-i**). Values are the mean ± SEM (n=3). Asterisks above the bars indicate one-way ANOVA statistical significance (***P < 0.001; ns = non significant) between the same varieties in different regions; letters above the bars indicate one-way ANOVA statistical significance (P< 0.05) between grapes from different varieties cultivated in the same region (upper case: north varieties, lower case: south varieties).

Changes in the total phenolics as accessed by Folin-Ciocalteau colorimetric method

The total phenolics evaluated in our laboratory along the grape berry development and ripening shows a very similar decreasing pattern in the three varieties from Dois Portos and EVAG (Figure 14a-c). At the maturity, the highest concentration value was found in grape berries from Padeiro de Basto cultivated in the North. Regarding the effect of sampling

region, the total phenolic content in grape berries from Arinto and Padeiro de Basto increased by 92% and 47%, respectively, from the South to the North (Figure 14e and f). In the Alvarinho cultivar the total phenolic content did not change significantly between the two regions.

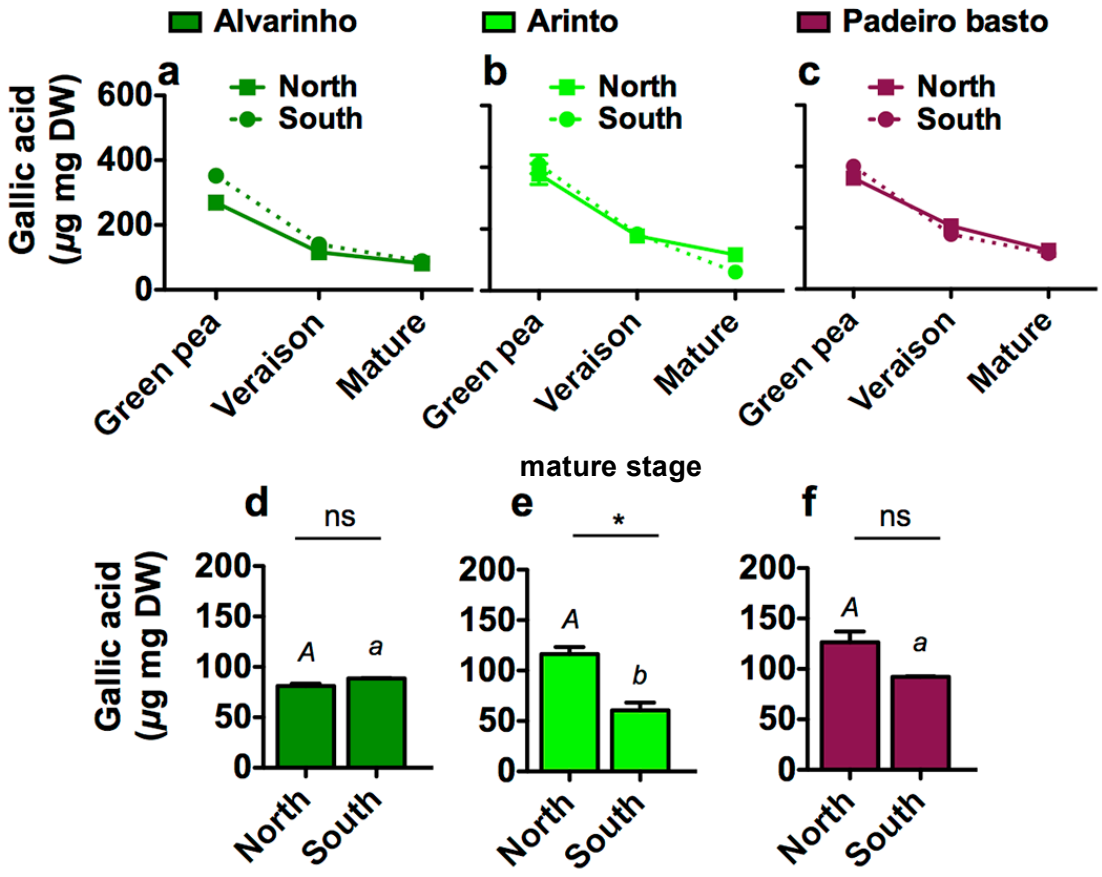


Figure 14. Total phenolics determined by Folin-Ciocalteau colorimetric method in grape berries from Alvarinho, Arinto and Padeiro de Basto cultivated in North (EVAG) and South (INIAV) Portugal**.** Results are expressed as microgram of gallic acid per miligram of berry DW. Values are the mean ± SEM (n=3). Asterisks above the bars indicate one-way ANOVA statistical significance (*P < 0.05; ns = non significant) between the same varieties cultivated in North and South; letters above the bars indicate one-way ANOVA statistical significance (P< 0.05) between the different varieties cultivated in the same region (upper case: north varieties, lower case: south varieties).

Changes in free amino acid content as assessed by an Aminoacid Analyser system

Previous GC-TOF-MS analysis detected thirteen amino acids in grape berries from all varieties from North and South (Supplementary Table 1). To complement these results, a quantitative approach was performed by an Aminoacid Analyser system for all amino acids, except tryptophan that was quantified by HPLC. The analysis was performed in grape berries

at the mature stage, and nineteen of the twenty free amino acids were detected and quantified. Cysteine was not detected in all samples.

As can be seen in Figures 15-17, arginine was the most abundant amino acid in all varieties and regions, eventually reflecting its role as precursor of the remaining amino acids. Proline and glutamic acid were also very abundant, with 504 and 502 mg/kg DW, respectively, in grape berries from Alvarinho in the North region.

The total amino acid content in the grape berries from each variety was as follows (in mg/Kg DW in North and South regions, respectively): Alvarinho, 4415 and 3056; Arinto, 3769 and 2340; and Padeiro de Basto, 3350 and 1406. Besides the observed genopype-dependent differences, data revealed that edaphoclimatic conditions also severely affected the content in amino acids in the grape berry. Thus, the concentration of free amino acids in grape berries is much higher in vines cultivated at EVAG than at Dois Portos (Figures 15e-17e). For instance, the total amino acid content in mature grapes from Padeiro de Basto increased by 138 % from the South to the North.

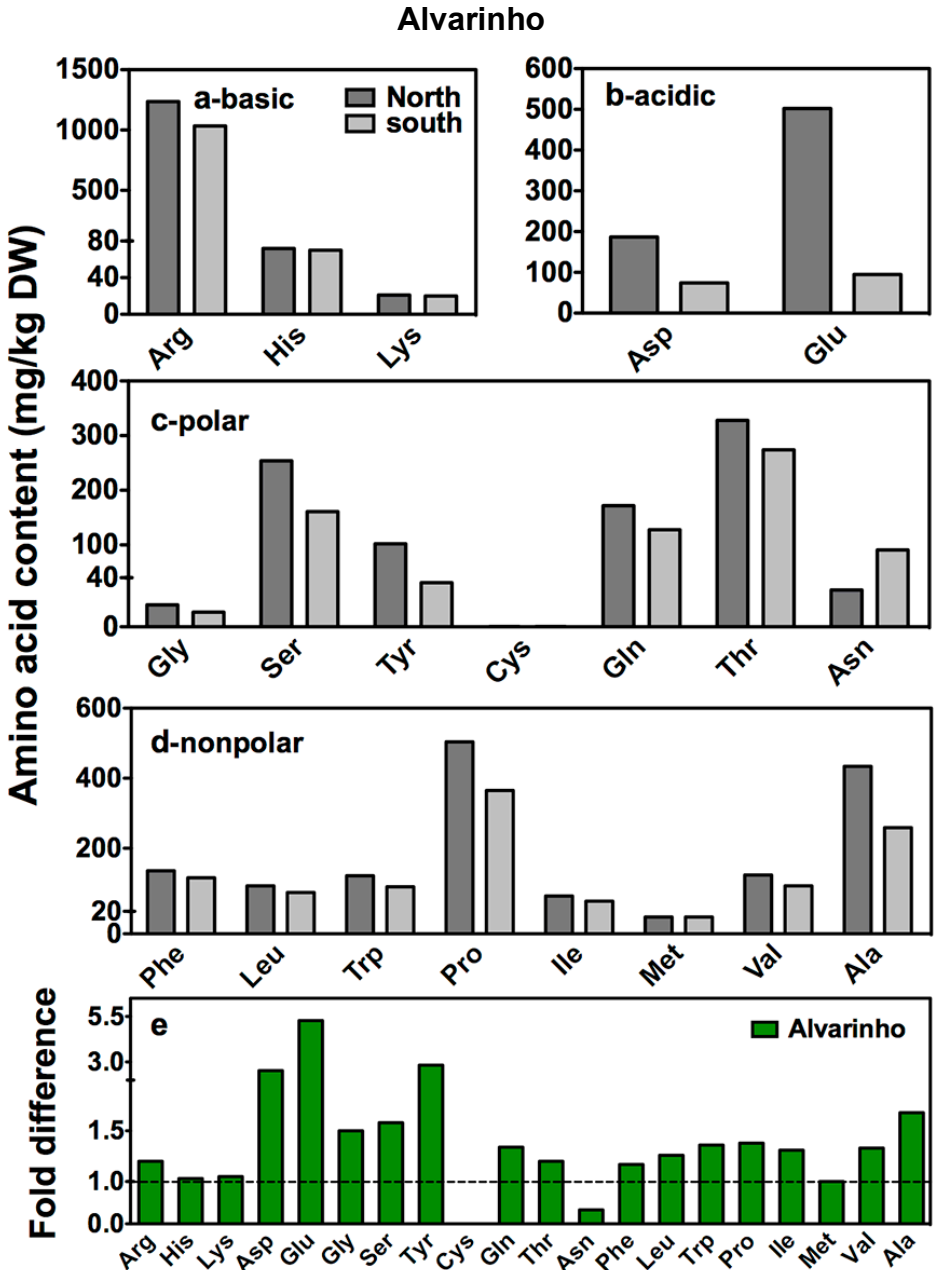


Figure 15. Free amino acid content as assessed by an Aminoacid Analyser system in grape berries from Alvarinho cultivated in North (EVAG) and South (INIAV) Portugal. The nineteen amino acids identified were grouped according its acidic/basic nature or charge characteristics: basic (a), acidic (b), polar (c), nonpolar (d). Concentration-fold difference between amino acids in mature grapes from Alvarinho cultivated in North and South regions (e).

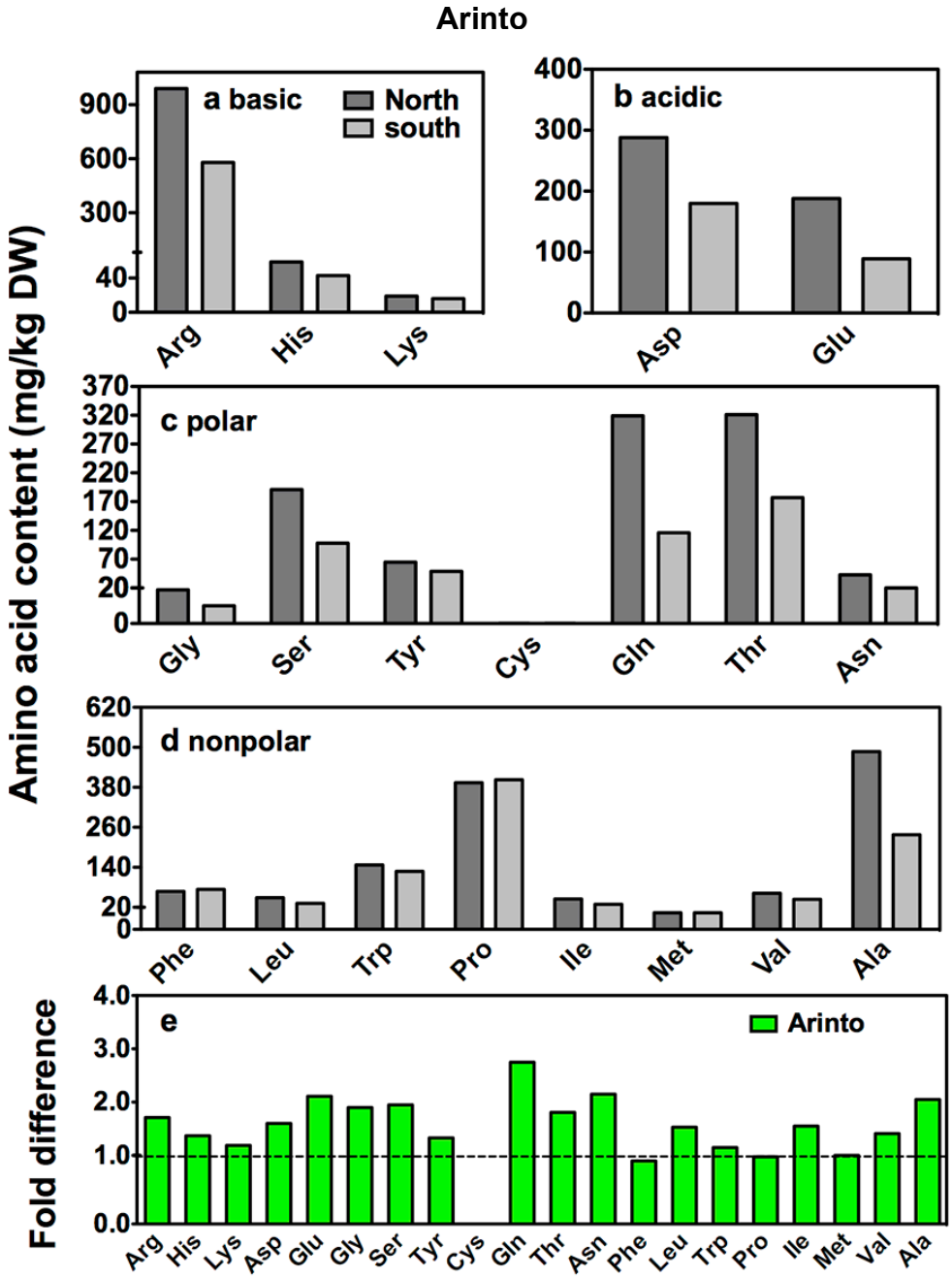


Figure 16. Free amino acid content as assessed by an Aminoacid Analyser system in grape berries from Arinto cultivated in North (EVAG) and South (INIAV) Portugal. The nineteen amino acids identified were grouped according its acidic/basic nature or charge characteristics: basic (a), acidic (b), polar (c), nonpolar (d). Concentration-fold difference between amino acids in mature grapes from Arinto cultivated in North and South regions (e).

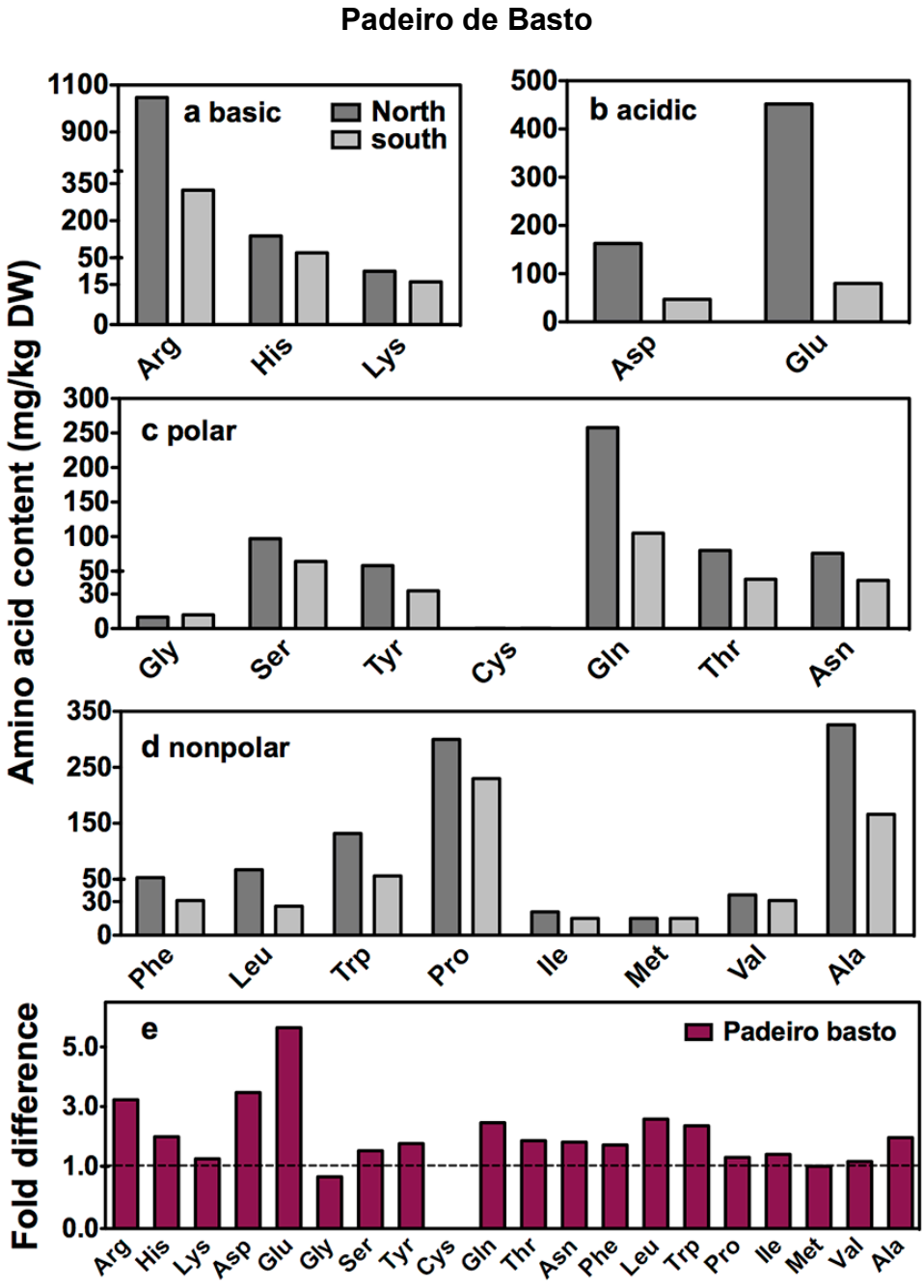


Figure 17. Free amino acid content as assessed by an Aminoacid Analyser system in grape berries from Padeiro de Basto cultivated in North (EVAG) and South (INIAV) Portugal. The nineteen amino acids identified were grouped according its acidic/basic nature or charge characteristics: basic (a), acidic (b), polar (c), nonpolar (d). Concentration-fold difference between amino acids in mature grapes from Padeiro de Basto cultivated in North and South regions (e).

4.3 Changes in key metabolic steps in grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal

Malate dehydrogenase (MDH) catalyses the reversible conversion of oxaloacetate (OAA) to malate maintaining the cytosolic equilibrium between these two key metabolic

intermediates. NADP-dependent malic enzyme (NADP-ME) catalyses the reversible conversion of malate to pyruvate, and, thus, it is also involved in malate metabolism, depending on the isoform involved, cellular conditions and the availability of substrates. MDH and NADP-ME activities were measured in cell extracts from whole berries at different developmental stages sampled from Alvarinho, Arinto and Padeiro de Basto varieties cultivated at INIAV (Dois Portos) and EVAG. As can be seen in Figure 18, the activity pattern of MDH during grape berry development and ripening seems to depend on the genotype and environment. In mature grape berries the highest activity of MDH was measured in Alvarinho cultivated in the North. From the North to the South there was a reduction in MDH activity by 49%. The remaining varieties also showed a reduction in MDH activity from the North to the South, although in Padeiro de Basto this variation was very short.

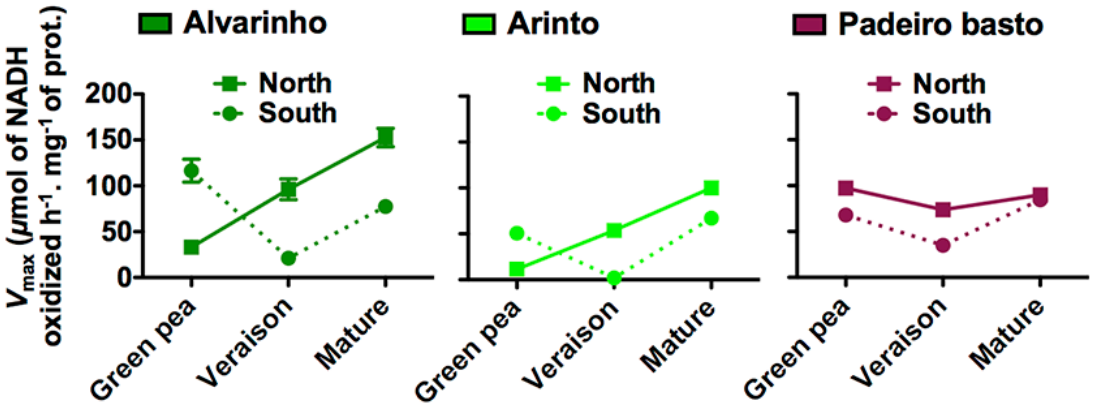


Figure 18. Malate dehydrogenase (MDH) activity in cell extracts of grape berries from Alvarinho, Arinto and Padeiro de Basto varieties cultivated in North (EVAG) and South (INIAV, Dois Portos) Portugal. Values are the mean ± SEM (n=3).

Figure 19 shows the activity pattern of NADP-ME during grape berry development and ripening in Alvarinho, Arinto and Padeiro de Basto cultivated at INIAV (Dois Portos) and EVAG. The most noticeable difference was observed in Arinto cultivar at the veraison stage, both in the North and South regions, when the activity of NADP-ME increased by 273% from the pea stage to the veraison before decreasing abruptly to 3.6 μmol h^{-1} mg $protein^{-1}$ at the mature stage. At maturity, the activity of NADP-ME was not very much different between varieties and sampling places.

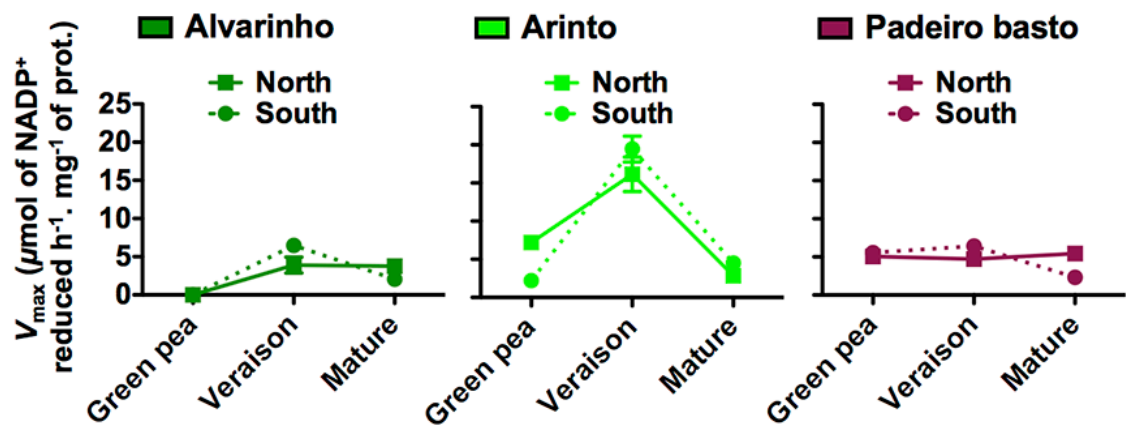


Figure 19. NADP-dependent malic enzyme activity (NADP-ME) in cell extracts of grape berries from Alvarinho, Arinto and Padeiro de Basto varieties cultivated in North (EVAG) and South (INIAV, Dois Portos) Portugal. Values are the mean ± SEM (n=3).

Mannitol dehydrogenases (VvMTDs) catalyses the conversion of mannitol to fructose and are likely to play important roles in polyol metabolism in grape berries. As in the case of MDH and NADP-ME, the total activity of MTD was measured in cell extracts from whole berries at different developmental stages sampled from Alvarinho, Arinto and Padeiro de Basto varieties cultivated at INIAV (Dois Portos) and EVAG. The biochemical activity of VvMTD steadily increased during ripening in grape berries from Padeiro de Basto vines cultivated in the North reaching its maximum rates of mannitol conversion to fructose (3.2 μmol h^{-1} mg $protein^{-1}$) at the mature stage (Figure 20c). Contrarily to the observed in Arinto and Padeiro de Basto cultivars, in grape berries from Alvarinho the activity of VvMTD decreases from veraison to the mature stage both in the South and in the North.

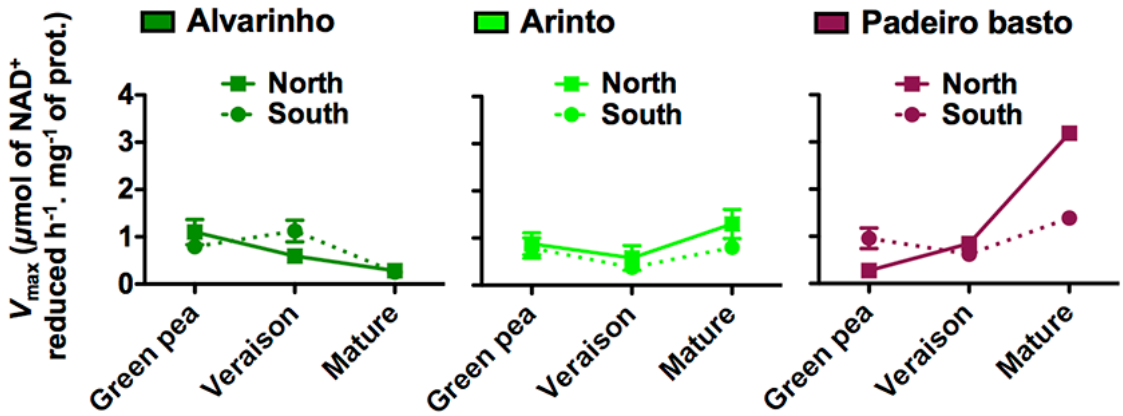


Figure 20. Mannitol dehydrogenase (MTD) activity in cell extracts of grape berries from Alvarinho, Arinto and Padeiro de Basto varieties cultivated in North (EVAG) and South (INIAV, Dois Portos) Portugal. Values are the mean ± SEM (n=3).

The enzyme cinnamate 4-hydroxylase is a cytochrome P450 that catalyses the oxidative reaction of *trans*-cinnamic acid into 4-hydroxy-cinnamate in a NADPH-dependent reaction. This compound has important antioxidative properties in wine. This reaction is one of the first steps of phenylpropanoids biosynthesis from phenylalanine, which leads to the production of several secondary metabolites. In Alvarinho and Padeiro de Basto there was a steadily decrease in C4H activity from green pea to mature stage. At the maturity, the activities of C4H in grape berries from the three varieties were lower in the South than in the North, particularly in Alvarinho and Padeiro de Basto where the activity of C4H decreased by 75% and 53% respectively (Figure 21d and f).

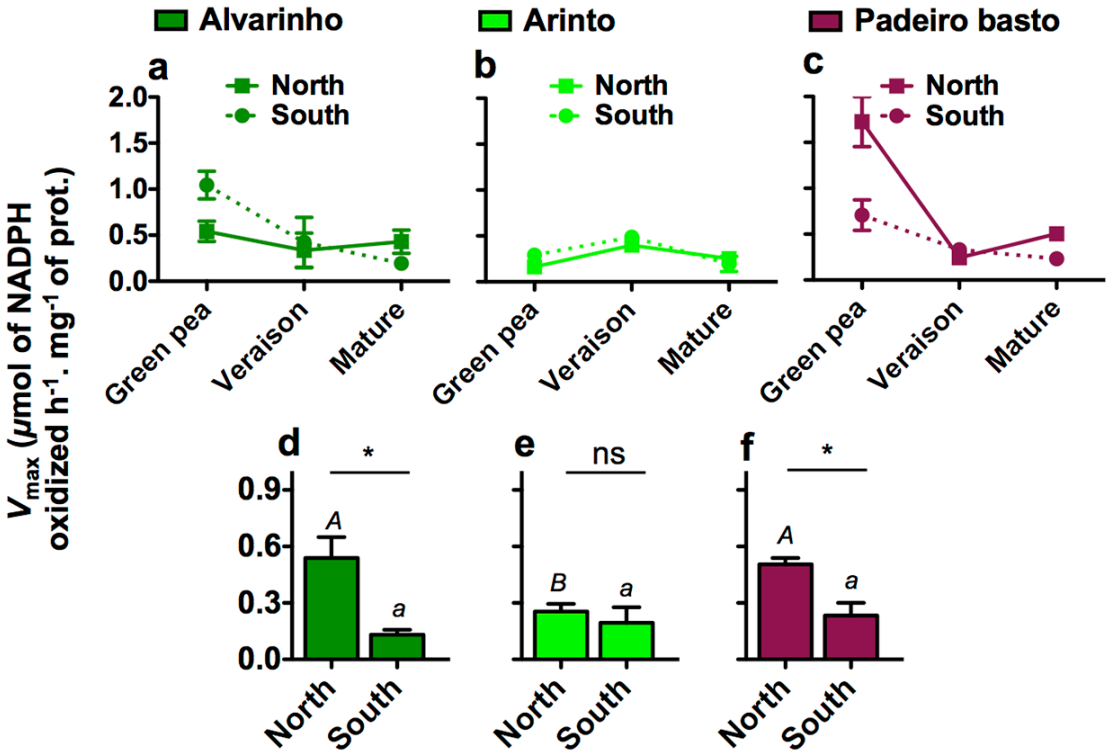


Figure 21. Cinnamate 4-Hydroxylase (C4H) activity in cell extracts of grape berries from Alvarinho, Arinto and Padeiro de Basto varieties cultivated in North (EVAG) and South (INIAV, Dois Portos) Portugal at green pea, veraison and mature stages (a - c). Activity of C4H at the mature stage in North (EVAG) and South (INIAV, Dois Portos) grapes. Values are the mean ± SEM (n=3). Asterisks above the bars denote one-way ANOVA statistical significance (*P < 0.05; ns = non significant) between the same varieties in different region; letters above the bars indicate one-way ANOVA statistical significance (P< 0.05) between the different varieties of the same region (upper case: north varieties, lower case: south varieties).

4.4 Total phenolics, antioxidant potential and proline content in mature berries from twenty-one grapevine varieties

Proline profile

As shown before, proline is one of the most abundant amino acids in berries, and may act as antioxidant and osmoprotectant in plant cells, besides its role as energy source. To investigate if proline levels in the grape berry could be a drought-stress resistance marker, its quantification was performed in mature grape berries from 21 Portuguese varieties from Dois Portos by the acid-ninhydrin colorimetric method. As shown in Figure 22, proline content is cultivar-dependent. Berries from the Tinto Cão red variety showed the highest proline concentration with 1734.5 mg/Kg FW followed by berries from the the white variety Airén with 1659.8 mg/Kg FW. In the red variety Corropio proline was not detected and was almost absent in Castelão with 51.3 mg/Kg FW. Although a genotype-dependence was clearly observed, results does not compare with those of the previous Section, probably because sampling was performed in different seasons and both the extraction and quantification methods were different.

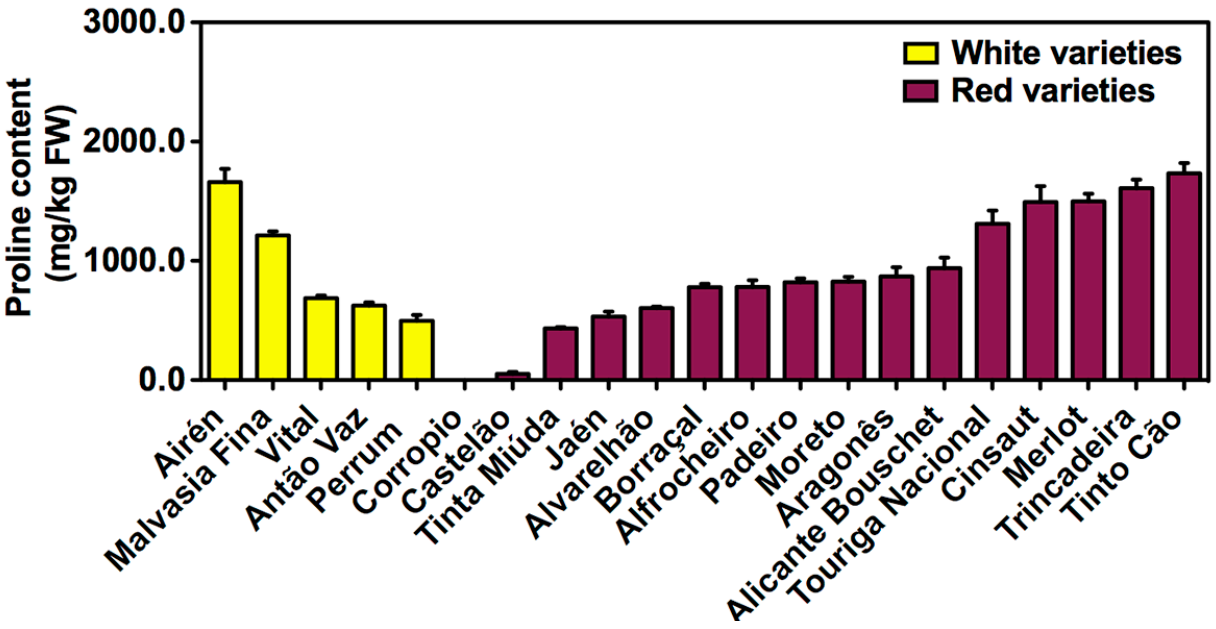


Figure 22. Free proline content in grape berries from twenty-one Portuguese grapevine varieties determined by the ninhydrin colorimetric method. Values are the mean ± SEM (n=3).

Total phenolic profile and antioxidant potential

As expected, mature grape berries from red varieties showed higher concentrations in total phenolics than white ones. Borraçal grapes reached values as high as 5.0 μg/mg FW. The

very important Portuguese grapevine cultivar, Touriga Nacional, showed also very high content in total phenolics.

As referred to in material and methods, the antioxidant capacity was evaluated with the method of Ferric Reducing Antioxidant Power (FRAP). As can be seen in Figure 23 (insert) there is a clear positive correlation between the antioxidant capacity of the grape berry extracts and their total phenolic content. Thus, as for total phenolic content, the highest antioxidant activity was found in grape berries from the red Borraçal variety while the lowest was found in the white grape berries from Airén variety.

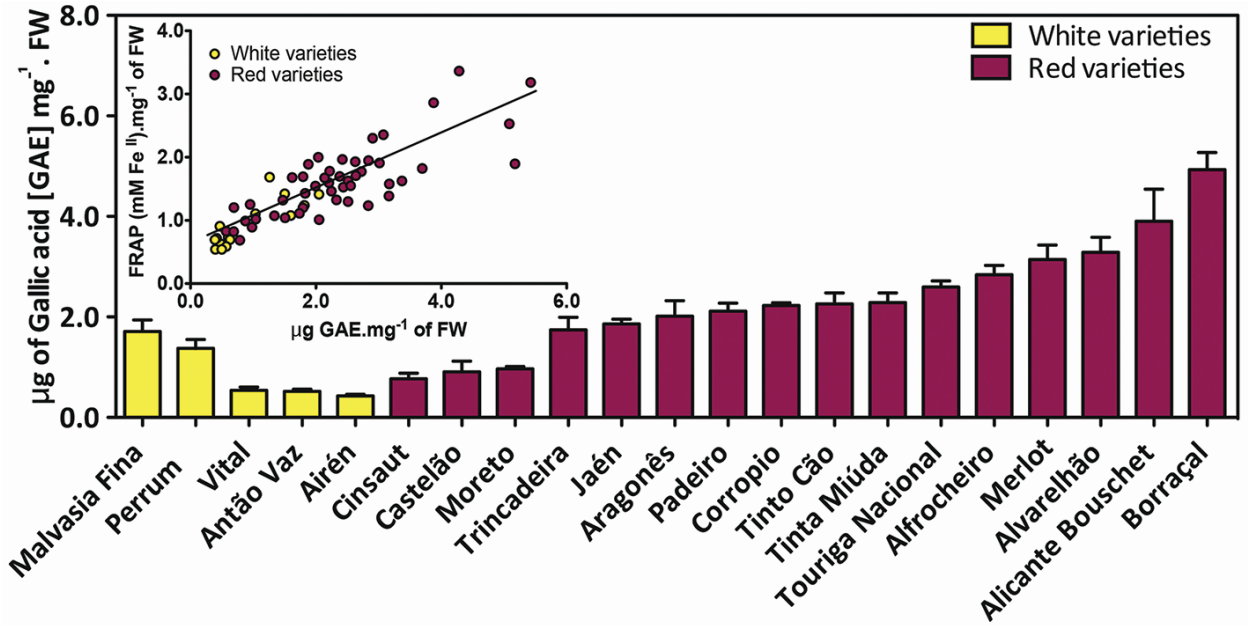


Figure 23. Total phenolics in mature grape berries from 21 Portuguese varieties determined by Folin-Ciocalteau method. Values are the mean ± SEM (n=3). Inset: Correlation between total phenolic content and antioxidant activity determined by the FRAP method.

4.5 Changes in the metabolism and metabolites in grape berries from vines infected with the GLRa V-3

Two infected cultivars were available at Dois Portos for these preliminary studies: Cabernet Sauvignon and Touriga Nacional. Mature grape berries from Cabernet Sauvignon cultivar infected with GLRaV-3 showed significant reduction in glucose and fructose concentrations, by 19% and 17%, respectively (Figure 24a and b), while the concentration of sucrose did not change from clean to infected plants. Contrarily, in grape berries from Touriga Nacional there was a significant reduction in sucrose concentration by 40% in infected plants

(Figure 24c), while glucose and fructose concentration did not change from clean to infected plants (Figure 24a and b).

The study of the relative expression of *VvHT1* and *VvHT6* in grapes from Touriga Nacional and Cabernet Sauvignon cultivars clean (control) and infected with GLRaV-3 was performed by RT-PCR. As shown in Figure 24d, *VvHT1* expression is repressed in Cabernet Sauvignon infected grapes, while *VvHT6* is up-regulated. In Touriga Nacional, the transcript levels of *VvHT1* are similar in berries from both clean and infected plants, while transcription of *VvHT6* is severely repressed in infected plants.

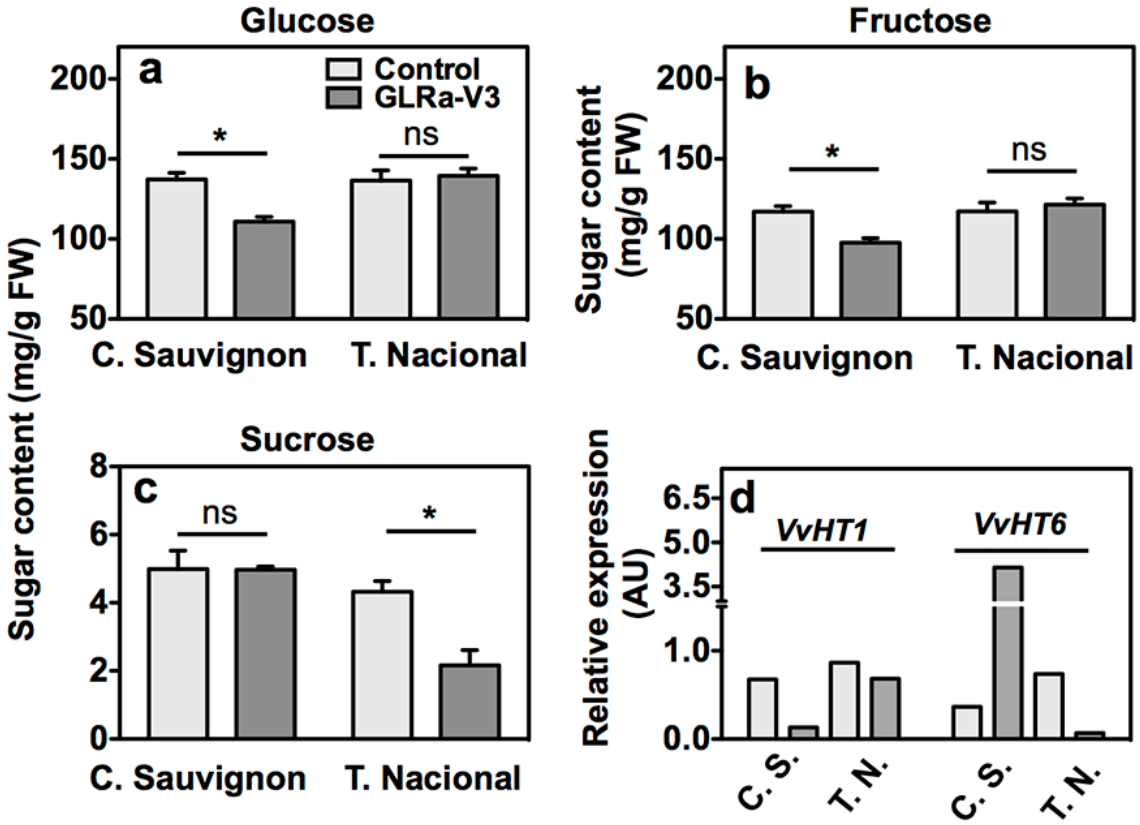


Figure 24. Sugar content in grape berries from Cabernet Sauvignon and Touriga Nacional cultivars clean (control) and infected with GLRa-V3, determined by HPLC (a-c). Transcript levels of *VvHT1* and *VvHT6* in berries from Cabernet Sauvignon and Touriga Nacional clean and infected with GLRaV-3 (d). Values are the mean ± SEM (n=3). Asterisks indicate one-way ANOVA statistical significance between control and infected grapevines (*P< 0.05; ns = non significant).

Virus infection did not cause a variation in the concentration of the sugar-alchool mannitol in grape berries from Cabernet Sauvignon (Figure 25a). Noticeably, this sugar alcohol was not detected in grape berries from the Touriga Nacional variety. As shown in Figure 25b, in berries from Cabernet Sauvignon MTD activity was lower in infected plants (0.36 μmol h^{-1} mg $protein^{-1}$) than in clean ones (0.58 µmol h^{-1} mg $protein^{-1}$) although this

reduction was not statistically significant. In Touriga Nacional, although this polyol was not detected in the berry, the specific activity of MTD was 0.43 μmol h^{-1} mg $protein^{-1}$ in both clean and infected plants.

Figure 25c shows that the concentration of free proline decreases after virus infection in grape berries from both grape varieties. This reduction in proline content was by 40% and 48% in infected Cabernet Sauvignon and Touriga Nacional cultivars, respectively.

Total phenols evaluated by Folin-Ciocalteau method showed a slight increase in berries from virus-infected Cabernet Sauvignon, while in Touriga Nacional there was a significant reduction in total phenols by 26% after virus infection (Figure 25d).

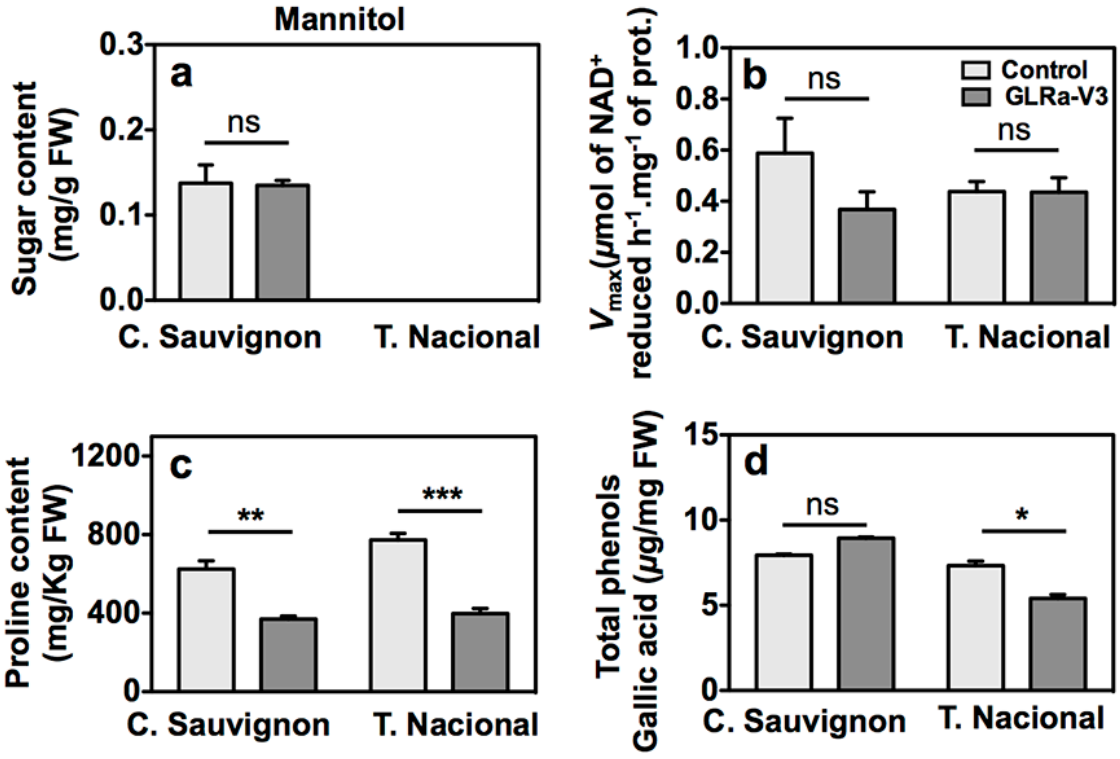


Figure 25. Manitol (a) and proline (c) content in berries from Cabernet Sauvignon and Touriga Nacional cultivars clean (control) and infected with GLRa-V3. Mannitol dehydrogenase (MTD) activity (b) and total phenolic content (d) determined by Folin-Ciocalteau method in grape berries from both cultivars before and after virus infection. Values are the mean ± SEM (n=3). Asterisks indicate one-way ANOVA statistical significance between control and infected grapevines. (*P< 0.05; **P< 0.01; ***P< 0.001; ns = non significant).

Anthocyanin levels were determined by a pH differential method, described by Nicoué et al. (2007). Figure 26a shows anthocyanin concentration in grape berries from clean and infected Cabernet Sauvignon and Touriga Nacional cultivars. As can be seen, the amount of anthocyanins decreased in response to virus infection in both grape varieties. It has been shown that phenylpropanoid pathway is affected by viral infection in a consistent way (Vega et al., 2011). Our results showed that the activity of C4H in grape berry extracts from both

infected cultivars was lower than in grapes from clean plants (Figure 26b). Additional experiments will be necessary to further consolidate these data.

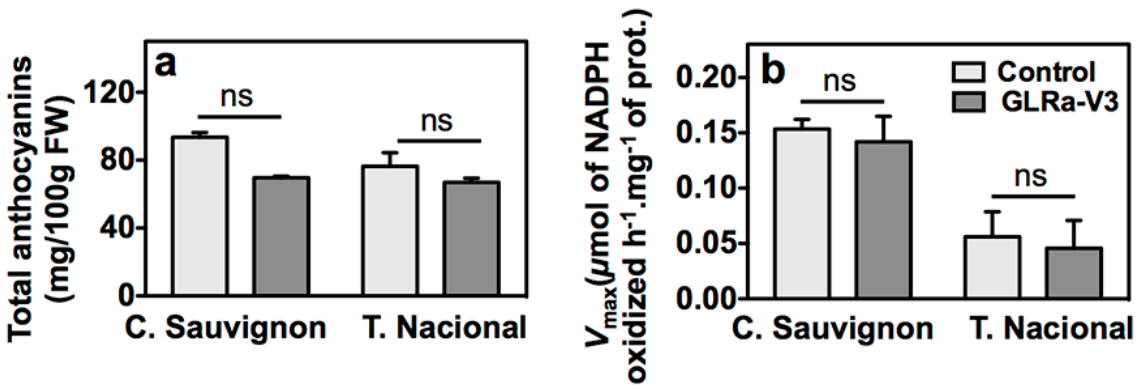


Figure 26. Total anthocyanin content (a) and cinnamate-4-hydroxylase (C4H) activity (b) in grape berries from Cabernet Sauvignon and Touriga Nacional cultivars clean (control) and infected with GLRa-V3. Values are the mean ± SEM (n=3). One-way ANOVA statistical significance between control and infected grapevine was calculated (ns = non significant).

5. Discussion

5 Discussion

5.1 The metabolome of berries from Alvarinho, Arinto, and Padeiro de Basto is dependent on the genotype and environment

Edaphoclimatic conditions, which may include temperature, precipitation, soil water content, composition and structure, evapotranspiration and relative humidity, clearly affected the metabolome profile of the grapes from Alvarinho, Arinto and Padeiro de Basto cultivars cultivated at Dois Portos (South Portugal) and EVAG (North Portugal). Also, genotype-dependent variations were evident. It should be stressed that the microclimate within the vineyard and cultural practices may greatly influence vine physiology and growth and the composition of the berries. These specific conditions were not considered in this work and may, per se, be object of independent studies. Also, clonal variations within each grape variety and season-dependent transcriptional changes (Santo et al., 2013) may also account for important biochemical variability in the grape composition.

It has been shown that sugar concentration in the berry is dependent on development stage (Coombe 1992), environment, and viticulture practices (Jackson and Lombard 1993, Kliewer and Dokoozlian 2005; Clingeleffer, 2010), as well as on the genotype (Shiraishi, 2000; Liu et al., 2006; Shiraishi et al., 2010). Contrarily, sugar concentration was considered a relatively stable trait for a given cultivar (Keller et al., 2005). In this context, a number of metabolomic analysis have been carried out to compare grape berry composition at various developmental stages (Ali et al., 2011), or looking at differences between cultivars and growing seasons (Pereira et al., 2006) or regions (Son et al., 2009). In our metabolome study, aimed to characterize three important Portuguese grape varieties - Alvarinho, Arinto and Padeiro de Basto -, approximately twenty different sugars were detected. Glucose, fructose and, in minor amounts, sucrose where the most important sugars in all varieties and regions, and sugar amount increased from *veraison* to the mature stage. Significant differences in sugar content, at the mature stage, between cultivars and regions were not expected, because maturity was considered when soluble solids reached 18 ºBrix for all varieties and regions. However, there was a very consistent difference between North and South in what regards sugar composition. The observed decrease of sucrose levels in the varieties cultivated in the South is very interesting and deserves further confirmation. This could be caused by stimulation of invertase activity by the warmer climate of Dois Portos. In agreement, a recent comparative analysis in tomato revealed that the activity of cell wall invertases (CWIN) may be modulated by heat (Li, et al. 2012).

Tartaric and malic acids typically account for 90% of total acids in grape berries. In our metabolome analysis, besides tartaric and malic acids, other organic acids, including maleic, citric, fumaric, and succinic were also detected in all samples but in relatively low amounts. In the non-climacteric fruit *Vitis vinifera,* malate metabolism has been a strong focus of research, as the balance of acids in winegrape must is central for supporting desirable growth (and preventing undesirable growth) of microorganisms responsible for wine fermentation. Malate concentration can also affect final wine characteristics through involvement in secondary processes such as carbonic maceration and malolactic fermentation, and can even alter the growth capabilities of malolactic bacteria (Kunkee, 1991). Since grapes do not contain large amounts of citrate, and the large quantity of tartrate present in the fruit is not used in primary metabolic pathways, malate is the only high-proportion organic acid that is actively metabolized throughout ripening of grapes (reviewed by Sweetman et al., 2009). It has been described that organic acids in the berry are responsive to environmental conditions and viticulture practices (Jackson and Lombard 1993, Keller et al., 2005). Our results confirmed that the amount of organic acids decreased during grape berry development and ripening after green pea stage, but the pattern of organic acid variation was similar between varieties. Pre-*veraison* grapes accumulate malate mostly through the metabolism of sugars that have been translocated to the berry, but also potentially through fruit photosynthesis (Hale, 1962, Breia et al., 2012). In post-*veraison* fruit, malate released from the vacuole becomes available for catabolism through various avenues, including the TCA cycle and respiration, gluconeogenesis, amino acid interconversions, ethanol fermentation, and the production of complex secondary compounds such as anthocyanins and flavonols (Farineau and Laval-Martin, 1977; Ruffner, et al. 1982b; Famiani et al., 2000). Interestingly, it has been reported that after *veraison* tartaric acid concentration decreases mostly through dilution due to volume increase of the fruit, but we observed a strong decrease in tartaric acid per dry weight of the berry, suggestion that it is substantially catabolized during ripening, at least in Alvarinho, Arinto and Padeiro de Basto.

Results also showed relatively high levels of malic acid and low levels of tartaric acid in mature grape berries from Alvarinho cultivated in the North when compared with two other varieties. Also, mature grapes from Alvarinho contained much less tartaric and malic acid in the South than in the North. Thus, the sampling place effectively affected the levels of tartaric and malic acid in the grape berries. In agreement, several reports emphasize that environmental factors affect malate concentration of the berries during ripening. Elevated temperature clearly decreases the concentration of malic acid, whereas grapevines grown in cool climates show higher amounts of malic acids (Keller et al., 2005; Koundouras et al.,

2006; Pereira et al., 2006). However, it has been described that tartaric acid concentration is not significantly affected by temperature or water stress (Parra et al., 2010), contrarily to the observed in our work.

Results showed that in all varieties and environments there was an increase of malate dehydrogenase (MDH) activity (Figure 18) from *veraison* to mature stage, which could be related with malic acid degradation (Figure 10). These results are in agreement with previous report by Taureilles-Saurel et al. (1995a). However the metabolism of malic acid is by far more complex. It is admitted that cytosolic and mitochondrial MDH isoforms would, respectively, participate in malate synthesis and catabolism in response to metabolic changes occurring during grape development (reviewed by Sweetman et al., 2009). However, it is admitted that the extraction and activity measurement conditions favors the cytosolic isoform of MDH (Taureilles-Saurel et al., 1995b). However, if cytosolic malate is transported and sequestered in the vacuole, then cytosolic MDH activity will be driven toward malate synthesis from OAA, until the equilibrium is re-established. Alternatively, if malate is abundant and OAA is further metabolized to compounds such as phosphoenolpyruvate (PEP) or aspartate, then MDH activity will favour the conversion of malate to OAA. It is in such (or similar) situations that MDH is henceforth suggested to be involved in malate ‘‘synthesis” or ‘‘degradation” (reviewed by Sweetman et al 2009). A reduction in MDH activity from the North to the South in mature grapes was evident in Alvarinho cultivar, however the levels of malic acid in mature grapes are lower in the south. Cool regions typically produce grapes with higher concentration of malic acid and, conversely, grapes grown in warmer regions tend to have lower acidity (reviewed by Conde et al 2007a). This negative temperature correlation with malic acid levels could be due to the effect of temperature on the balance between malic acid synthesis and catabolism.

Both the variety and environment seemed to influence NADP-dependent malic enzyme activity pattern during grape berry development, but a correlation between malic acid levels and enzyme activity is difficult to establish due to the complexity of the organic acid metabolism where MDH, NADP-dependent malic enzyme, phosphoenolpyruvate carboxykinase (PEPCK) and phosphoenolpyruvate carboxylase (PEPC) may be involved. Some transcriptional studies on organic acid metabolic enzymes have been reported so far (Deluc et al., 2007, Sweetman et al., 2011). The study of these enzymes at both the transcriptional and at the protein activity levels may shed some light to the metabolic pathways of organic acids, which are highly intermingled.

It has been shown that the accumulation of compatible solutes consisting of non-toxic organic molecules, such as polyols protects the cells against deleterious osmotic and metabolic imbalances caused by stress (Conde et al 2011a; Pillet et al., 2012; Conde et al. unpublished). The involvement of polyols in abiotic stress plant tolerance has been a fierce research topic throughout the years. Mannitol is the most widespread polyol in nature and has been observed in >100 vascular plant species of several families including the Apiaceae (celery, carrot and parsley), Rubiaceae (coffee) and Oleaceae (olive and privet) (Lewis, 1984). In olive and celery, mannitol synthesis takes place in mature leaves from mannose-6-phosphate by the conjugated action of a NADPH-dependent mannose-6-phosphate reductase (M6PR) and a mannose-6-phosphate phosphatase, and is then translocated through the phloem to heterotrophic sink tissues where it can be either stored or oxidized to mannose *via* the action of a 1-oxireductase, NAD^+-dependent mannitol dehydrogenase (MTD), and used as a carbon and energy source (reviewed by Stoop et al., 1996; Noiraud et al., 2001b). Sorbitol is synthesized in mature leaves from glucose-6-phosphate by the consecutive activity of an aldose-6-P-reductase (Negm and Loescher 1981) and a specific phosphatase, and, generally, sinks have little or no capacity to synthesize polyols like mannitol or sorbitol (Loescher and Everard 1996; Nawodnik and Lohaus 2008). However, in grapevine, the knowledge on the metabolism of polyols is very limited. In our laboratory siginificant efforts have been done to understand plant response to drought throught the production of polyols, in the context of the PhD dissertations of Paulo Silva (2013) and Artur Conde (2013). The metabolome analysis of the present study identified sorbitol, ribitol, galactinol, erythritol in grape berries from all varieties, and sorbitol was the most abundant polyol in mature berries from Padeiro de Basto. Regarding the effect of edaphoclimatic conditions, it was suggested that the levels of polyols were generally higher in the varieties cultivated in the South, eventually because they were subjected to more intense heat and drought stress than the vines cultivated in the North. Mannitol was not detected, but we confirmed that berry tissues from all varieties showed MTD activity, eventually to convert mannitol to fructose.

Total amino acid content is known to vary between cultivars and according microclimate conditions in response to sun exposure (Cantagrel et al., 1962; Henschke and Jiranek, 1992; Pereira et al., 2006). Several factors may affect nitrogen nutrition of grapevines, such as vine cultivar and rootstock, climate and season, N levels in the soil, cultural practices, canopy shading and microclimate. Grapevines are able to absorb both NO_3^- and NH_4^+ ions from the soil. The reduction of NH_3^- is started by nitrate reductase, forming NO_2^- which is then reduced to NH_4^+ by nitrite reductase in the chloroplast. Besides other

fates, one of the main roles of NH_4^+ is the incorporation into amino acids. In the present study we decide to further investigate how the amino acid content changes between the Portuguese cultivars Alvarinho, Arinto and Padeiro de Basto from one cultivating region to another. As shown, GC-TOF-MS qualitative analysis (Supplementary Table 1) performed at the Genome Center (UC Davis) only detected twelve amino acids in all grape berry samples, but the quantitative approach requested to Ansynth Service B.V. performed with an amino acid analyzer and HPLC provided information about all twenty free amino acids in the mature grapes. Due to time and economic constrains, only one analysis was performed, however, to minimize the error due to the absence of biological replicates three equal parts of lyophilized powder from one different cluster from each vine (3 vines) were mixed before analysis. We showed that the mature grape berry is full of amino acids. From the twenty free amino acids, only cysteine was not present. Arginine, proline and glutamic acid were the most abundant. In agreement, it has been shown that proline and arginine are the two most abundant amino acids in the musts (Kliewer, 1970; Huang and Ough, 1989; 1991; Asensio et al 2002). Proline may contribute to a sweet taste in the berry, and some biological functions are attributed to this amino acid that include acting as an energy source, antioxidant and osmoprotectant (Coruzzi et al. 2000; Forde et al., 2007; Deluc et al., 2009). Previous experiments showed that water deficit significantly increased proline concentrations in Cabernet Sauvignon berries but not in Chardonnay (Deluc et al., 2009). In our results, proline concentration was higher in Alvarinho and Padeiro de Basto cultivars in the North region, with more abundant levels of water in the soil. Alvarinho (North) was the variety with higher amino acid content in the mature berries. The pattern of amino acid content did not change very much between genotypes, but edaphoclimatic conditions severely affected the content of amino acids, which is much higher in the North in all varieties. In this regard, the amino acid content in pulp and skin of Merlot showed different patterns in response to sun exposure (Pereira et al; 2006). Because the levels and type of aminoacids may significantly influence the secondary aromas of wines (Hernandez-Orte, Cacho, and Ferreira, 2002), aroma analisys by GC-MG of micro-fermentations with berries from North and Southn could bring interesting outputs to the wine scientific community.

Grape secondary compounds have been for many years a theme of major scientific and biotechnological interest. Grape berry phenolics contribute to organoleptic properties, colour and protection against environmental challenges. This issue was extensively explored in Section 2, where environmental changes and genotype-dependent modifications on phenolic content were subject of particular attention. The qualitative metabolomic profile of

the three grape varieties in both regions revealed few phenolic compounds as only some hydroxycinnamates, catechin and epicatechin were detected. Future studies may relay on LC-MS analysis and different extraction protocols to more accurately study grape berry secondary metabolites.

Benzoic acid is the precursor of several common hydroxybenzoic acids, usually found in wine, such as gallic acid, gentisic acid, *p*-hydroxybenzoic acid, protocatechuic acid (3,4-Dihydroxybenzoic acid), syringic acid, salicylic acid, and vanillic acid (Peña-Neira et al., 2000; Pozo-Bayón et al., 2003; Monagas et al., 2005a). We found that benzoic acid that was much more abundant in mature grape berries from Alvarinho and Arinto cultivated in the South. However, 3,4-dihydroxybenzoic acid showed a decreasing pattern in all varieties from both regions along the development and similar AU levels at mature stage.

The acid 3,4-dihydroxybenzoic is involved in several reactions as phenylalanine, tyrosine and tryptophan biosynthesis, toluene degradation, polycyclic aromatic hydrocarbon degradation, aminobenzoate degradation or biosynthesis of phenylpropanoids (KEGG - Kyoto Encyclopedia of Genes and Genomes; Kanehisa and Goto, 2000). In an extensive screening of 344 European grape cultivars for two consecutive years, hydroxybenzoic acids concentration at the mature stage was in average 0.016 mg g^{-1} FW (Chen et al., 2006). It was also showed that hydroxybenzoic acid concentration was highly variable from one year to another, contrarily to the observed for other phenolic compounds such as anthocyanins, flavanols, hydroxycinnamic acids, or flavonols. It was suggested that phenolic content of grape berries is highly variable, and that each phenolic compounds may change differently in response to environmental factors. In agreement, we showed that the levels of caffeic acid seemed to be slightly lower in the grape berries from the varieties cultivated in the South than in the North, especially in Padeiro de Basto, contrarily to benzoic acid.

It was reported flavan-3-ols compounds account for 36% of total non-anthocyanin polyphenols and procyanidin B1 is the most abundant flavanol in grape berry, accounting for 64% of total flavanols. Catechins account on average for 20% of total flavanols, epicatechin and epicatechin gallate account for no more than 10% of total flavanols (Chen et al 2006). In our metabolomic approach only (+) catechin and its isomer (-) epicatechin were identified. Both catechins and epicatechins significantly decreased from the North to the South suggesting that their levels in mature grapes are dependent on the growing region.

Because the analysis performed at the UC Davis was by far incomplete regarding secondary metabolites, we decided to study changes in the total phenolics by the Folin-Ciocalteau colorimetric method. It was observed that total phenolic concentration, measured as gallic acid equivalents, decreased in all varieties and regions from the green to the mature

stage, contrasting with previous results (Matthews et al., 1988; Zarrouk et al., 2012). At maturity, the highest concentration value was found in grape berries from Padeiro de Basto cultivated in the North, probably due to the contribution of anthocyanins. In Arinto and Padeiro de Basto varieties, total phenolics were much higher in the North than in the South. These results were quite unexptected because vines from the South may suffer more intense environmental stresses, incluing water limitation and drought. In agreement, in Tempranillo grapevines subjected to drought conditions, total phenolic content of the skin was higher in RDI and NI vines when it was compared to FI during two consecutive years (Zarrouk et al., 2012). However, the sun exposure of grape clusters can influence the flavanol content of grape berries. In a previous study it was shown that total phenolics content in grapes from shaded clusters was lower than in grapes from clusters exposed or moderately exposed to the sun (Price et al., 1995).

Gene expression and post-transcriptional modifications of several enzymes of the secondary metabolism may depend on the genotype and environment. Secondary metabolism begins from the condensation of phosphoenolpyruvate (PEP; from the glycolysis pathway) and erythrose 4-phosphate (E4P; from the pentose phosphate pathway) to produce 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) by the action of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), the first step of shikimate pathway. This pathway is defined as seven metabolic steps ending with the synthesis of chorismate, the precursor of the aromatic amino acids, tyrosine, tryptophan and phenylalanine, substrates for downstream enzymes of the phenylpropanoid pathway. The phenylpropanoid pathway is of pivotal importance in secondary plant metabolism, where a myriad of phenolic secondary metabolites unique to plants are produced (phenolic acids, flavonoids, lignins, and stilbenes). The enzymes phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate: coenzyme A ligase (4CL) are considered to be crucial to phenylpropanoid metabolism (Weisshaar and Jenkins, 1998; Brenda, 1999). Thus, the transcript abundance of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DHPS), chorismate mutase and PAL was significantly increased in water deficit in Cabernet Sauvignon, however, the transcript levels of these genes were not increased by water deficit in Chardonnay. In a similar way, the shikimate concentration was increased by water deficits in Cabernet Sauvignon, but not in Chardonnay (Deluc et al., 2009). In the present study, the region seemed to affect the activity of C4H in mature grapes from Alvarinho and Padeiro de Basto as a very significant reduction occurred in the South region. This variation may explain, at least in part, the observed reduction in some secondary metabolites in the South (catechin and epicatechins, as shown by GC-TOF-MS) and total phenolics in Arinto and Padeiro de Basto, as show by Folin-

Ciocalteau colorimetric method. Some reports show the activity of PAL, C4H and 4CL during development and ripening of the grape berry (Hrazdina et al., 1984; Chen et al., (2006) but there is no clear picture about their expression and activity pattern during development. Still, there is litle information regarding the effect of environment x genotype on the activity of these enzymes. So, the data of the present work open good perspectives to further explore the influence of the environment and genotype on secondary metabolism in the grape berry. In addition, recently available high-throughput RNA sequencing approaches will likely help to elucidate in a near future the complexity of secondary metabolism in the grape berry in challenging environments.

5.2 Total phenolics, proline content and antioxidant potential are genotype-dependent traits

In the second part of this project we aimed at exploring in more detail the genotype-dependency of specific markers of berry quality, including proline content, total phenolics, and antioxidant activit. For that purpose a set of 21 Portuguese varieties were selected from the germplasm collection of the Estação Agronómica Nacional (Dois Portos) and grape samples were collected at the mature stage, as described in Material and Methods.

The levels of proline content are clearly different between varieties. Tinto Cão berries had the highest proline concentration, while in Corropio proline was not detected. As referred before, this amino acid may act as antioxidant and osmoprotectant in plant cells, besides its role as energy source (Coruzzi et al 2000; Forde et al. 2007; Deluc et al. 2009). Several reports show that increases in proline concentration are one of the most common responses of organisms to dehydration (Yancey et al. 1982; Delauney and Verma 1993; Cramer et al 2007). As referred above, water deficit significantly increased proline concentrations in Cabernet Sauvignon berries with no significant effect in Chardonnay. Given that proline levels in the grape berry may represent a drought-stress resistance marker further investigation is necessary to evaluate the real capacity of Tinto Cão, Malvazia Fina, Trincadeira and Touriga Nacional to resist to water limitation more than Corropio or Castelão. Besides the remarkable cultivar-dependence, care has to be taken when comparing these results with those of the previous Section, because two different methods were used in grapes from different seasons (2011 and 2012).

Regarding the total phenolic content, as expected, mature grape berries from red varieties showed higher concentrations in total phenolics than white ones, due to the contribution of anthocyanins that accumulate in red varieties at the mature stage. Thus,

Alicante Bouschet, which is a teinturier, and Borraçal, whith a thick and dark exocarp, exhibited the highest amount of total phenolics and, consequently, higher antioxidant activity. These analyses are relatively easy to perform and could be extended to characterize all Portuguese varieties, particularly including seeds analysis, because they have been used in important sectors, including oil production and in cosmetics industry due to their strong antioxidant capacity. Thus, the ongoing work may have an important biotechnological dimension enabling farmers and enologists to select specific varieties to prepare wines with a desired antioxidant activity.

5.3 The metabolism of the grape berries from Touriga Nacional and Cabernet Sauvignon is modified in grapevines infected with the GLRa V-3 virus

Leafroll has long known effects on grapevine maturity and berry pigmentation (Goheen, 1970). Our results showed that the metabolism of the grape berries is altered in Cabernet Sauvignon and Touriga Nacional infected with GLRaV-3. Although these studies were preliminary, it was shown that virus infection modified sugar accumulation and sugar transporter expression. Several monossacharide (VvHT) and disschariде transporters (VvSUC) cooperate in sugar transport across the plasma membrane of grape cells whose expression may be affected by environmental conditions and sugar status of the berry (Kuhn et al., 1997; Conde et al., 2006). In the present study it was not observed a significant change in glucose and fructose amounts in the berries from clean and infected vines of Touriga Nacional, although the *VvHT1* transcription sufered a significant reduction. Contrarily, in Cabernet Sauvignon, a significant reduction of glucose and fructose was observed. These results are in agreement with a previous study in the same variety infected with GLRa-V3. The authors showed that *VvHT1* and its transcription factor *MSA* mRNA levels were significantly repressed in virus-infected grape berries (Vega et al., 2011), which was consistent with decreased amounts of glucose and fructose found in mature berries. Regarding the putative tonoplast transporter *VvHT6*, our results showed that it was upregulated in infected vines of Cabernet Sauvignon and severely repressed in infected vines from Touriga Nacional. To verify possible causes of virus-induced alterations of some photosynthetic parameters, the expression level of some genes involved in sucrose metabolism in cv. Merlot were previously evaluated (Repetto et al. 2012). Expression levels of SuSy (sucrose synthase), CWINV (cell wall invertase) and five genes encoding neutral invertases (NI 1-5) were measured. Interestingly, transcript profiles of six INV and one SuSy did not reveal any difference in gene expression level between virus-infected and non-infected plants (Repetto et

al., 2012). From the available data, we may conclude further studies are still necessary to complete the puzzle regarding the metabolic changes induced by GLRa V-3 virus in grapevine.

As discussed before, the involvement of polyols in abiotic stress plant tolerance has been a fierce research topic throughout the years, however, the role of polyols on plant response to abiotic stress is far from being understood. In our study only mannitol was detected by HPLC in Cabernet Sauvignon whose levels were not modified in infected vines. In berries from Touriga Nacional mannitol was not detected suggesting that its acumulation is cultivar dependent.

The knowledge on the modification of the activity of metabolic enzymes upon virus infection in grapevine is for while very short. Our results showed that the activity of mannitol dehydrogenase (MTD) was not significantly modified upon infection of both cultivars. However, MTD activity decreased in berries from Cabernet Sauvignon upon infection, but more replicates are necessary to confirm this result. It is known that phenylpropanoid pathway is affected by viral infection in a consistent way (Vega et al., 2011), however our study showed that cinnamate-4-hydroxilase (C4H) activity was not significantly modified upon virus infection. Future studies should also focus on the activity of phenylalanine ammonia lyase (PAL) that converts L-phenylalanine or tyrosine into *trans*-cinnamic acid.

Regarding proline, the present study showed that its concentration is lower in berries from infected than in clean plants. As report before (Lee et al., 2009) the levels of valine, methionine and glutamate in Pinot Noir response to virus infection may depend on the location of the vineyard but, in general, GLRaV infection did not greatly impact N-containing compounds. Contrarily, more arginine and less proline at harvest were found in berries from infected Burger grapes with GLRaV than in healthy plants (Kliewer and Lider, 1976). Therefore, more investigation is needed to clarify the influence of virus infection on amino acid metabolism in the berry, including proline biosynthesis and degradation.

To the best of our knowledge, very few papers (Gutha et al., 2010) describe alterations of phenylpropanoid pathway in response to GLRa-V3 infection. It was reported that 17 flavonoid biosynthetic pathway genes showed higher expression levels in virus-infected symptomatic leaves than in virus-free plants. Among them, *CHS3, F3'5'H, F3H1, LDOX* and *LAR1* showed more than 10-fold increase in leaves from virus-infected plants. In the present study we found a slightly decrease of cinnamate-4-hydroxylase activity and in the amount of anthocyanins in grape berries from clean to virus-infected plants, but the results are very preliminary. In agreement, more recent reports postulated an anthocyanin reduction yield in GLRa-V3 infected grapevines (Vega et al., 2011). The repression of some key genes in

anthocyanin biosynthesis has been observed in response to virus infection (Gutha et al., 2010).

5.4 Final considerations and future perspectives

The physiology of grapevine has already suffered from significant impacts of global climate change over the last decades. In this context, the study of the influence of edaphoclimatic conditions in the metabolomic profile of grape berries is of utmost importance to help the farmers select the proper genotype and to adjust the agricultural practices in order to keep good levels of productivity and maximizing wine quality. This topic was the main target of our research.

Grape berries from three important Portuguese wine varieties from two distinct ampelographic collections, in Northern (EVAG) and Southern (INIAV-Dois-Portos) Portugal, were analysed with up-to-date approaches and equipments, including GC-TOF-MS and the Amino Acid Analyser System. Edaphoclimatic conditions clearly affected the metabolome profile of the grape berries from Alvarinho, Arinto and Padeiro de Basto cultivars. Thus, it was observed that mature grapes from Alvarinho contained much less tartaric and malic acid in the South than in the North, and that grape berries from the North are much richer in aminoacids. It is well known that organic acids are essential for the fresh character of the wine and free amino acids may play import role in the aroma development.

In addition, several cultivar-dependent traits were identified in this work. For instance, sorbitol and proline were very abundant in grapes from Padeiro de Basto and Tinto Cão, respectively.

Biotic stresses, including fungal and virus infections, also negatively impact grapevine physiology and productivity, causing significant economic losses every year. The preliminary studies developed in this context, confirmed that grape berries metabolism is altered in Cabernet Sauvignon and Touriga Nacional infected with GLRaV-3, but the puzzle regarding the metabolic changes induced by GLRa V-3 virus still needs further investigation.

Several “omics” approaches, including genomic, transcriptomic and metabolomic, are now being explored in several laboratories worldwide to study and mitigate the influence of biotic and abiotic stresses in grapevine productivity, in the context of the ongoing climate changes. Also, the exploitation of grapevine genetic diversity is of utmost importance to select cultivars more adapted to a specific region or microlimate. As referred to before, the investment in new varieties that would give good flavors but with improved climate tolerances may be an important investment for the industry and for conservationists wishing

to avoid unfavorable land or water use outcomes.

Several laboratories and institutions of our country, including our own at Universidade do Minho, the CITAB researcher unit (UTAD), the Instituto Superior de Agronomia (ISA), the Insituto de Tecnologia e Química e Biológica (ITQB) and Instituto Nacional de Investigação Agrária e Veternária (INIAV), among others, are working together to study the grapevine physiology/echophysiology and fruit maturation and composition at the biochemical and molecular levels, and to explore/preserve the tipicity or our important cultivars, including Touriga Nacional, Arinto, Padeiro de Basto, and Alvarinho, among many others. The active involvement of our research teams in several European Cooperation Actions (COST) and National and International European projects, such as Innovine, is promoting important progresses at the scientific level with important repercussions in the agronomic sector.

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7 Supplementary files

7.1 Metabolomic profile

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

Carbohydrate metabolism and glycolysis													
	Metabolite	Stage*	Alvarinho				Arinto				Padeiro de Basto			
			North		South		North		South		North		South	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
1	sucrose	G	**31913**	1587	**1192**	57	**22028**	55	**1321**	72	**38302**	405	**431**	77
		V	**84120**	2986	**23329**	261	**56769**	1617	**16893**	525	**69028**	1895	**14928**	98
		M	**146376**	727	**23372**	1314	**102057**	9859	**67126**	73	**123143**	9192	**29961**	382
2	glucose	G	**99509**	1821	**88904**	578	**105312**	28	**121881**	3764	**116848**	1457	**92160**	177
		V	**517015**	8247	**314704**	1173	**484160**	3340	**378761**	4873	**575225**	2324	**539029**	8520
		M	**566069**	3132	**557481**	7239	**609915**	43094	**615787**	7921	**734059**	34156	**606181**	17545
3	fructose	G	**39513**	610	**44198**	132	**56012**	293	**58680**	1448	**56300**	25	**44350**	71
		V	**1607560**	117535	**1296052**	4992	**1617848**	52406	**1258729**	46720	**583464**	16814	**1670545**	92695
		M	**611684**	7250	**611345**	8516	**1628774**	407852	**623696**	25295	**739760**	50995	**591713**	29971
4	xylulose NIST	G	**541**	92	**1451**	354	**399**	20	**984**	153	**1975**	184	**3052**	186
		V	**155**	31	**179**	18	**157**	4	**171**	13	**263**	26	**278**	4
		M	**143**	9	**179**	19	**152**	22	**173**	183	**213**	54	**199**	42
5	inulotriose	G	**320**	18	**186**	17	**177**	27	**218**	39	**228**	29	**150**	13
		V	**443**	49	**219**	51	**288**	35	**244**	39	**332**	19	**285**	28
		M	**474**	75	**353**	34	**456**	54	**567**	29	**485**	28	**406**	25
6	fucose + rhamnose	G	**975**	146	**842**	88	**1045**	37	**1377**	461	**888**	271	**627**	78
		V	**715**	60	**646**	85	**1300**	15	**1165**	3	**741**	85	**794**	93
		M	**640**	16	**628**	61	**680**	81	**865**	215	**549**	135	**537**	47

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

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Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

			Carbohydrate metabolism and glycolysis											
			Alvarinho				**Arinto**				**Padeiro de Basto**			
	Metabolite	**Stage***	North		South		North		South		North		South	
			mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
7	**arabinose**	G	**703**	36	**1368**	110	**1070**	92	**1615**	583	**627**	66	**1221**	236
		V	**309**	14	**267**	42	**344**	58	**449**	18	**298**	21	**331**	35
		M	**275**	36	**254**	32	**242**	76	**264**	29	**231**	64	**228**	50
8	**beta-gentiobiose**	G	**359**	16	**322**	38	**295**	10	**354**	79	**352**	46	**308**	21
		V	**837**	47	**326**	35	**471**	31	**504**	209	**1557**	28	**698**	71
		M	**1218**	19	**744**	126	**499**	126	**680**	40	**3036**	851	**2046**	66
9	**glycero-guloheptose NIST**	G	**618**	22	**882**	85	**744**	51	**1039**	16	**563**	85	**446**	25
		V	**557**	46	**487**	13	**847**	40	**848**	76	**388**	60	**358**	38
		M	**516**	22	**617**	37	**767**	83	**881**	54	**602**	61	**488**	80
10	**levanbiose**	G	**192**	21	**199**	65	**237**	22	**216**	74	**183**	12	**182**	14
		V	**2379**	143	**1175**	88	**3229**	225	**1770**	77	**2782**	164	**2145**	128
		M	**2740**	67	**2215**	250	**2615**	573	**3538**	94	**2607**	648	**2551**	79
11	**glycerol-3-galactoside**	G	**1130**	37	**1743**	42	**799**	21	**1068**	874	**771**	56	**1127**	33
		V	**433**	33	**523**	22	**667**	24	**1135**	60	**547**	51	**917**	31
		M	**217**	25	**770**	86	**330**	54	**497**	91	**396**	128	**591**	56
12	**rhamnose**	G	**982**	75	**1073**	180	**861**	54	**1426**	420	**1300**	7	**827**	109
		V	**734**	209	**734**	48	**877**	35	**995**	60	**901**	279	**883**	133
		M	**642**	18	**1101**	490	**734**	117	**984**	112	**581**	163	**543**	51
13	**3,6-anhydrogalactose**	G	**977**	33	**2196**	64	**1228**	71	**1686**	108	**1236**	22	**1991**	33
		V	**673**	21	**163**	19	**111**	5	**351**	274	**572**	49	**126**	21
		M	**135**	40	**107**	11	**128**	33	**144**	26	**125**	36	**132**	15
14	**sorbitol**	G	**311**	35	**372**	13	**322**	30	**314**	257	**308**	26	**313**	8
		V	**670**	739	**704**	21	**915**	481	**626**	444	**246**	66	**70**	54
		M	**300**	54	**563**	148	**168**	21	**282**	111	**2206**	658	**2485**	165

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

			Carbohydrate metabolism and glycolysis											
	Metabolite	**Stage***	**Alvarinho**				**Arinto**				**Padeiro de Basto**			
			North		South		North		South		North		South	
			mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
15	**ribitol**	G	**210**	36	**233**	51	**173**	35	**297**	67	**132**	65	**166**	63
		V	**174**	33	**163**	2	**169**	16	**173**	12	**165**	29	**178**	15
		M	**222**	37	**163**	20	**150**	74	**164**	167	**139**	38	**162**	27
16	**galactinol**	G	**6594**	252	**5634**	250	**4677**	215	**8524**	2202	**3100**	261	**2985**	170
		V	**1139**	128	**994**	95	**1997**	43	**3506**	428	**1045**	71	**2447**	158
		M	**673**	101	**1051**	227	**1168**	213	**1716**	68	**962**	216	**1379**	84
17	**erythritol**	G	**177**	24	**379**	102	**262**	24	**386**	79	**274**	21	**278**	17
		V	**558**	111	**362**	89	**577**	53	**806**	51	**568**	77	**701**	87
		M	**941**	45	**930**	146	**591**	140	**832**	46	**662**	104	**718**	65
18	**1,5-anhydroglucitol**	G	**286**	79	**326**	47	**2260**		**342**	70	**289**	32	**214**	24
		V	**2680**	57	**1938**	170	**769**	89	**578**	79	**892**	14	**717**	53
		M	**854**	56	**777**	35	**3762**	1418	**2076**	76	**1193**	25	**660**	46
19	**conduritol beta epoxide**	G	**3223**	14	**2119**	82	**3612**	78	**3754**	45	**2762**	85	**1978**	37
		V	**3140**	58	**1401**	32	**3192**	83	**3868**	85	**2758**	84	**2298**	12
		M	**3020**	62	**1909**	91	**2986**	118	**3128**	51	**2589**	91	**2729**	65
20	**levoglucosan**	G	**428**	61	**199**	65	**237**	22	**216**	74	**183**	12	**182**	14
		V	**223**	4	**1175**	88	**3359**	37	**1770**	77	**2782**	164	**2076**	57
		M	**205**	30	**2085**	153	**2915**	341	**3538**	94	**2952**	354	**2551**	79

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

Continued

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

			TCA cycle and other acids											
			Alvarinho				Arinto				Padeiro de Basto			
	Metabolite	Stage*	North		South		South		North		North		South	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
21	tartaric acid	G	**218819**	9687	**339258**	713	**325248**	4735	**335119**	41010	**322475**	2717	**292950**	808
		V	**48821**	726	**41213**	215	**76480**	2073	**86611**	1887	**88020**	1249	**97759**	1438
		M	**32707**	91	**15615**	135	**39972**	25	**37183**	668	**50529**	368	**36286**	427
22	malic acid	G	**448984**	8024	**397530**	6036	**359020**	3824	**366609**	17489	**408478**	1215	**300821**	2614
		V	**222137**	7145	**258947**	4217	**304769**	7503	**518102**	11539	**212593**	5111	**247296**	4075
		M	**110346**	2098	**71910**	1666	**93831**	17557	**80549**	3629	**85315**	17413	**64009**	1081
23	citric acid	G	**22943**	276	**18021**	226	**18368**	65	**20286**	503	**23769**	297	**17391**	353
		V	**21450**	564	**15657**	493	**28150**	656	**51943**	1662	**16690**	618	**16698**	412
		M	**13643**	505	**10977**	74	**13660,5**	44,5	**13367**	341	**7876**	1780	**7669**	204
24	fumaric acid	G	**1534**	73	**1381**	75	**1194**	109	**913**	78	**1067**	42	**954**	45
		V	**3758**	122	**981**	117	**3396**	111	**7924**	513	**1569**	120	**4623**	222
		M	**488**	55	**2187**	98	**264**	50	**502**	22	**272**	65	**729**	47
25	maleic acid	G	**90455**	3630	**57873**	2293	**52111**	675	**62387**	4145	**72013**	2194	**60514**	1319
		V	**238465**	11137	**137729**	3744	**192553**	7169	**477995**	16352	**139651**	3584	**291279**	3584
		M	**25731**	428	**93122**	2104	**36034**	49966	**37627**	1076	**37489**	52499	**51244**	946
26	succinic acid	G	**1358**	1	**1047**	51	**1205**	25	**1041**	115	**1394**	51	**915**	15
		V	**370**	10	**135**	41	**466**	70	**570**	54	**251**	55	**432**	18
		M	**185**	63	**254**	39	**462**	128	**371**	35	**263**	49	**180**	22
27	ribonic acid	G	**889**	40	**1269**	123	**540**	44	**1211**	323	**1529**	55	**1092**	40
		V	**195**	31	**285**	28	**176**	29	**160**	18	**177**	46	**204**	65
		M	**193**	44	**318**	67	**173**	49	**214**	214	**126**	73	**167**	46
28	stearic acid	G	**16778**	703	**15311**	260	**12360**	190	**13905**	31	**13499**	476	**16771**	155
		V	**11640**	89	**18598**	344	**16244**	300	**13622**	462	**17588**	382	**20461**	399
		M	**11225**	71	**19336**	207	**18246**	1538	**20158**	510	**24131**	18	**13194**	863
29	benzoic acid	G	**4418**	77	**894**	68	**753**	32	**3247**	303	**3436**	39	**2896**	14
		V	**3157**	61	**1168**	34	**4159**	37	**3922**	95	**3355**	6	**4382**	119
		M	**755**	2	**3942**	68	**1406**	85	**4835**	23	**985**	73	**927**	48
30	aspartic acid	G	**3471**	187	**1710**	69	**2336**	172	**875**	112	**5150**	96	**894**	121
		V	**1478**	45	**1723**	58	**3098**	124	**3517**	143	**1691**	38	**1033**	70
		M	**1863**	44	**515**	14	**3092**	31	**2048**	16	**1191**	165	**439**	22

Continued on next page - * G, Green pea; V, *veraison*; M, Mature

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

			TCA cycle and other acids											
			Alvarinho				Arinto				Padeiro de Basto			
	Metabolite	Stage*	North		South		South		North		North		South	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
31	lactic acid	G	**2491**	80	**1029**	38	**956**	64	**1831**	33	**1623**	532	**1569**	104
		V	**2218**	37	**773**	71	**1935**	62	**1690**	62	**1783**	37	**1474**	44
		M	**637**	51	**1652**	29	**1836**	151	**2266**	49	**944**	64	**786**	87
32	glutamic acid	G	**2542**	66	**2081**	76	**1944**	84	**1149**	158	**2442**	21	**850**	9
		V	**913**	22	**596**	24	**660**	99	**1142**	40	**774**	23	**468**	68
		M	**2506**	35	**405**	50	**980**	47	**436**	91	**2308**	42	**336**	20
33	oxalic acid	G	**7006**	554	**4129**	16	**7308**	60	**5052**	639	**5493**	116	**3923**	33
		V	**1827**	387	**635**	56	**1256**	8	**1118**	100	**1363**	288	**1968**	179
		M	**1267**	35	**1124**	313	**2738**	157	**6277**	705	**3452**	315	**945**	121
34	myristic acid	G	**295**	0	**335**	22	**323**	19	**285**	43	**307**	9	**347**	9
		V	**236**	84	**194**	25	**179**	42	**193**	64	**208**	74	**196**	57
		M	**193**	23	**312**	65	**221**	71	**241**	21	**227**	44	**160**	13
35	lauric acid	G	**1088**	83	**1313**	30	**814**	54	**917**	101	**1020**	54	**1132**	20
		V	**851**	80	**1184**	23	**786**	79	**910**	54	**728**	43	**911**	49
		M	**323**	14	**1205**	60	**590**	66	**1087**	25	**662**	61	**724**	123
36	lactobionic acid	G	**507**	28	**536**	48	**455**	32	**499**	126	**498**	27	**405**	15
		V	**483**	10	**323**	44	**203**	49	**381**	18	**204**	28	**193**	38
		M	**123**	37	**382**	19	**146**	13	**364**	23	**181**	75	**151**	26
37	glucuronic acid	G	**594**	86	**847**	58	**742**	40	**1738**	419	**637**	41	**871**	60
		V	**230**	28	**252**	62	**230**	32	**179**	46	**163**	20	**177**	69
		M	**159**	16	**197**	13	**149**	42	**160**	19	**156**	48	**184**	11
38	galactonic acid	G	**192**	54	**329**	8	**220**	28	**394**	102	**355**	12	**320**	8
		V	**295**	18	**313**	21	**227**	11	**161**	10	**174**	20	**219**	26
		M	**382**	28	**308**	80	**253**	46	**378**	66	**237**	52	**263**	25
39	digalacturonic acid	G	**265**	5	**286**	49	**265**	7	**510**	15	**197**	18	**229**	31
		V	**204**	24	**153**	23	**205**	51	**312**	24	**158**	13	**178**	23
		M	**169**	16	**169**	14	**177**	28	**144**	27	**177**	62	**151**	26
40	dehydroascorbic acid	G	**5816**	175	**2113**	50	**3829**	35	**3060**	146	**9148**	98	**2546**	67
		V	**1787**	27	**686**	15	**1876**	80	**503**	37	**1848**	51	**799**	113
		M	**1593**	24	**540**	39	**1271**	13	**1025**	25	**1663**	37	**849**	104

Continued on next page - * G, Green pea; V, *veraison*; M, Mature

Continued

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

TCA cycle and other acids

	Metabolite	Stage*	Alvarinho				Arinto				Padeiro de Basto			
			North		South		South		North		North		South	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
41	2-ketogulonic acid	G	**11259**	104	**18726**	91	**3948**	54	**7786**	64	**5951**	69	**9033**	70
		V	**188**	22	**605**	54	**169**	15	**313**	25	**158**	56	**166**	26
		M	**146**	21	**143**	19	**146**	31	**139**	43	**187**	49	**131**	2
42	3,4-dihydroxybenzoic acid	G	**1244**	40	**1409**	78	**1812**	7	**1492**	77	**2327**	23	**1002**	22
		V	**467**	5	**401**	16	**618**	91	**464**	83	**335**	55	**386**	46
		M	**342**	22	**298**	43	**255**	39	**222**	46	**245**	48	**348**	48
43	3-hydroxypropionic acid	G	**915**	177	**245**	12	**260**	11	**548**	86	**462**	19	**369**	39
		V	**446**	18	**159**	4	**528**	66	**551**	40	**415**	22	**544**	50
		M	**122**	3	**486**	49	**338**	66	**640**	14	**189**	82	**126**	10

Amino acid and nitrogen metabolism

	Metabolite	Stage*	Alvarinho				Arinto				Padeiro de Basto			
			North		South		North		South		North		South	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
44	citrulline	G	**186**	32	**167**	27	**156**	13	**124**	18	**157**	20	**158**	20
		V	**1996**	60	**516**	17	**957**	344	**685**	47	**714**	55	**361**	21
		M	**1529**	127	**1542**	99	**1141**	281	**802**	223	**607**	177	**675**	79
45	ethanolamine	G	**20514**	130	**20992**	491	**22239**	439	**23266**	2039	**17721**	346	**16600**	369
		V	**4697**	121	**3752**	65	**6126**	184	**7136**	77	**4917**	96	**5360**	127
		M	**1644**	161	**4579**	224	**2854**	480	**4591**	319	**2545**	606	**5155**	167
46	hydroxylamine	G	**200**	105	**160**	15	**164**	54	**137**	45	**207**	128	**120**	24
		V	**515**	77	**135**	26	**343**	184	**249**	85	**166**	59	**125**	22
		M	**360**	175	**462**	38	**170**	68	**151**	33	**131**	61	**327**	121
47	ornithine	G	**140**	13	**112**	21	**128**	12	**115**	23	**164**	66	**134**	24
		V	**1049**	152	**300**	79	**600**	99	**407**	127	**433**	60	**173**	14
		M	**768**	83	**254**	123	**479**	145	**303**	66	**232**	59	**149**	15

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

			Alvarinho				Arinto				Padeiro de Basto			
			North		South		North		South		North		South	
	Metabolite	**Stage***	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
	Amino acid and nitrogen metabolism													
		G	**17219**	5	**21098**	318	**12582**	188	**6712**	194	**12335**	130	**7468**	118
48	**oxoproline**	V	**2656**	58	**1103**	62	**2561**	166	**2879**	70	**2026**	83	**850**	83
		M	**2481**	114	**994**	37	**2249**	462	**1363**	360	**2355**	624	**972**	50
		G	**2792**	141	**2827**	42	**4563**	41	**3622**	207	**8472**	132	**3879**	111
49	**putrescine**	V	**829**	66	**533**	26	**863**	24	**881**	34	**1112**	78	**963**	145
		M	**754**	108	**447**	273	**916**	233	**557**	193	**979**	139	**1453**	70
		G	**1380**	244	**1635**	185	**1516**	529	**1753**	432	**1692**	183	**1531**	346
50	**sarcosine**	V	**1986**	167	**1866**	126	**1861**	167	**1697**	52	**1629**	165	**1573**	225
		M	**1727**	21	**1643**	27	**1760**	349	**1767**	52	**1734**	490	**1452**	125
		G	**35818**	1329	**52362**	1025	**89963**	359	**148681**	33501	**87738**	131	**38109**	283
51	**shikimic acid**	V	**2487**	6	**4440**	48	**1546**	106	**2314**	139	**1782**	46	**3358**	92
		M	**5855**	196	**4162**	111	**16603**	596	**15191**	203	**5359**	225	**5305**	294
		G	**504**	64	**141**	25	**129**	40	**133**	86	**404**	11	**121**	23
52	**urea**	V	**525**	34	**408**	48	**505**	47	**494**	58	**296**	129	**133**	18
		M	**412**	37	**119**	22	**618**	131	**487**	23	**467**	146	**606**	23

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

Continued

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

Amino acids

	Metabolite	Stage*	Alvarinho				Arinto				Padeiro de Basto			
			North		South		North		South		North		South	
			mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
53	**proline**	G	**1434**	56	**1483**	57	**953**	59	**1400**	82	**997**	136	**1123**	163
		V	**5467**	52	**1792**	4	**2629**	67	**3179**	107	**2368**	161	**1725**	36
		M	**10926**	465	**6028**	66	**9604**	902	**9382**	169	**5992**	972	**4482**	545
54	**asparagine**	G	**968**	54	**1174**	11	**525**	78	**835**	549	**888**	20	**930**	58
		V	**362**	22	**522**	15	**274**	3	**329**	38	**550**	36	**296**	29
		M	**255**	24	**225**	89	**334**	81	**232**	58	**303**	61	**207**	7
55	**glutamine**	G	**23201**	561	**21806**	136	**13598**	190	**6953**	589	**14111**	1243	**8558**	195
		V	**2480**	53	**894**	85	**2012**	30	**1884**	44	**1169**	52	**452**	19
		M	**793**	29	**600**	28	**1633**	81	**483**	25	**744**	42	**489**	78
56	**glycine**	G	**765**	8	**869**	50	**728**	42	**852**	77	**560**	9	**625**	53
		V	**654**	64	**341**	39	**642**	69	**807**	39	**420**	10	**231**	25
		M	**466**	13	**459**	34	**718**	119	**600**	55	**368**	93	**496**	14
57	**isoleucine**	G	**494**	41	**642**	22	**440**	40	**498**	71	**467**	37	**461**	79
		V	**994**	40	**497**	16	**823**	59	**849**	34	**526**	87	**259**	30
		M	**1180**	10	**950**	63	**909**	61	**593**	20	**458**	96	**347**	28
58	**leucine**	G	**660**	6	**1135**	69	**813**	59	**721**	480	**688**	77	**702**	101
		V	**1628**	7	**557**	46	**959**	44	**1014**	15	**1819**	124	**113**	4
		M	**2093**	52	**1642**	91	**1322**	81	**964**	42	**1213**	125	**505**	29
59	**phenylalanine**	G	**980**	36	**911**	11	**1315**	68	**1550**	101	**637**	32	**635**	97
		V	**848**	22	**432**	24	**563**	31	**1284**	80	**2173**	1271	**866**	175
		M	**1100**	16	**822**	95	**524**	83	**658**	14	**812**	107	**371**	90
60	**serine**	G	**7917**	2	**8862**	52	**5880**	149	**7073**	954	**7108**	399	**7208**	405
		V	**4708**	126	**1757**	91	**3329**	167	**3021**	90	**1650**	104	**829**	65
		M	**3335**	90	**2162**	4	**2973**	303	**1920**	91	**1277**	70	**719**	59
61	**threonine**	G	**2294**	286	**2300**	162	**1834**	18	**2611**	100	**1633**	144	**1875**	179
		V	**3947**	128	**1904**	15	**3163**	76	**2124**	36	**1657**	286	**828**	214
		M	**4413**	60	**3188**	242	**4778**	303	**3650**	6	**1510**	313	**1125**	121
62	**tryptophan**	G	**938**	128	**888**	38	**436**	17	**760**	9	**1550**	80	**680**	37
		V	**2807**	107	**1557**	43	**2903**	136	**2643**	74	**1913**	77	**1467**	108
		M	**2323**	76	**1948**	123	**3343**	106	**2780**	88	**2081**	70	**1199**	130

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

Amino acids

	Metabolite	Stage*	Alvarinho				Arinto				Padeiro de Basto			
			North		South		North		South		North		South	
			mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
63	**tyrosine**	G	**555**	107	**697**	63	**600**	4	**719**	2	**556**	26	**560**	37
		V	**1981**	77	**515**	58	**1444**	58	**1138**	62	**1508**	35	**505**	78
		M	**2737**	93	**737**	5	**2073**	156	**1325**	85	**1196**	21	**504**	93
64	**valine**	G	**2202**	171	**2764**	2	**2092**	35	**2456**	278	**2050**	14	**1567**	163
		V	**2765**	63	**817**	42	**1451**	19	**1544**	73	**737**	93	**301**	3
		M	**3460**	42	**2449**	90	**1982**	183	**1595**	105	**1134**	115	**801**	49

Glyoxylate metabolism, signaling and others

	Metabolite	Stage*	Alvarinho				Arinto				Padeiro de Basto			
			North		South		North		South		North		South	
			mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
65	**FAD**	G	**1030**	19	**2556**	93	**1389**	39	**2232**	556	**1335**	41	**2324**	115
		V	**125**	7	**159**	24	**119**	17	**199**	19	**128**	27	**137**	26
		M	**119**	11	**124**	13	**131**	29	**137**	11	**134**	17	**109**	20
66	**GABA**	G	**29661**	326	**16129**	292	**16764**	157	**14141**	1161	**18342**	592	**12755**	313
		V	**13025**	626	**6981**	121	**14101**	121	**19185**	402	**11416**	168	**8625**	387
		M	**4639**	25	**11997**	11	**8746**	1492	**9419**	465	**6117**	483	**14786**	103
67	**glyceric acid**	G	**835**	69	**1259**	43	**133**	4	**752**	69	**228**	190	**510**	13
		V	**1371**	61	**1280**	47	**953**	120	**965**	57	**927**	52	**599**	37
		M	**106**	11	**250**	227	**148**	23	**111**	9	**145**	29	**110**	27
68	**idonic acid NIST**	G	**4796**	174	**6936**	245	**2279**	36	**4133**	1037	**3390**	92	**3462**	49
		V	**4355**	176	**5248**	41	**1324**	43	**880**	77	**1269**	89	**2005**	51
		M	**4475**	126	**5119**	216	**1180**	255	**1884**	93	**1350**	110	**1494**	90
69	**phosphoric acid**	G	**7020**	705	**9487**	607	**5164**	232	**8747**	2214	**3414**	508	**5175**	388
		V	**3612**	171	**3235**	146	**5004**	212	**6286**	237	**3639**	373	**4146**	342
		M	**3607**	300	**4950**	91	**4050**	631	**6103**	302	**4643**	356	**3546**	205

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

Continued

			Glyoxylate metabolism, signaling and others											
	Metabolite	Stage*	Alvarinho				Arinto				Padeiro de Basto			
			North		South		North		South		North		South	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
70	pipecolic acid	G	**525**	70	**633**	105	**159**	37	**134**	12	**200**	32	**206**	19
		V	**610**	28	**523**	24	**203**	18	**224**	22	**229**	33	**129**	3
		M	**681**	34	**402**	24	**206**	39	**205**	13	**316**	35	**184**	19
71	propane-1,3-diol NIST	G	**1061**	57	**1077**	51	**1046**	71	**1112**	303	**1025**	42	**1216**	249
		V	**1099**	50	**1022**	83	**1193**	33	**1144**	139	**1082**	70	**1129**	66
		M	**1070**	69	**1075**	71	**1198**	240	**1117**	59	**1200**	112	**1057**	301
72	quinic acid	G	**1402**	163	**2259**	26	**1146**	42	**2318**	645	**1031**	46	**944**	58
		V	**329**	39	**473**	8	**392**	28	**373**	18	**187**	5	**213**	24
		M	**220**	7	**216**	16	**257**	62	**389**	15	**153**	43	**165**	18
73	lyxose	G	**449**	92	**1117**	1305	**621**	78	**2554**	2674	**805**	113	**710**	90
		V	**439**	54	**353**	69	**724**	211	**824**	83	**542**	601	**807**	130
		M	**284**	5	**267**	100	**687**	144	**824**	49	**381**	37	**461**	118
74	ribose	G	**170**	21	**279**	75	**113**	97	**352**	62	**214**	28	**236**	21
		V	**74**	38	**151**	6	**195**	17	**182**	19	**176**	12	**129**	60
		M	**176**	20	**59**	8	**178**	13	**127**	82	**165**	31	**81**	38
75	1-monostearin	G	**111**	25	**138**	26	**146**	8	**113**	5	**127**	39	**107**	3
		V	**118**	24	**686**	24	**123**	22	**122**	9	**99**	5	**107**	18
		M	**120**	7	**128**	14	**115**	23	**114**	15	**123**	66	**150**	18

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

			Oxidative stress											
	Metabolite	**Stage***	**Alvarinho**				**Arinto**				**Padeiro de Basto**			
			North		South		North		South		North		South	
			mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
76	**dehydroascorbic acid**	G	**5816**	175	**2113**	50	**3769**	106	**3060**	146	**8996**	271	**2546**	67
		V	**1727**	106	**686**	15	**1821**	111	**503**	37	**1904**	103	**837**	104
		M	**1593**	24	**540**	39	**1146**	216	**1025**	25	**1464**	345	**849**	104
77	**threonic acid**	G	**15350**	23	**24303**	340	**19867**	216	**28681**	6722	**16732**	536	**18807**	237
		V	**2487**	6	**4440**	48	**1546**	106	**2314**	139	**1782**	46	**3358**	92
		M	**699**	9	**1463**	80	**427**	77	**491**	8	**1097**	253	**805**	71

			Lipid metabolism											
	Metabolite	**Stage***	**Alvarinho**				**Arinto**				**Padeiro de Basto**			
			North		South		North		South		North		South	
			mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
78	**myristic acid**	G	**295**	0	**335**	22	**323**	19	**285**	43	**307**	9	**347**	9
		V	**236**	84	**194**	25	**179**	42	**193**	64	**166**	2	**196**	57
		M	**193**	23	**312**	65	**221**	71	**241**	21	**227**	44	**160**	13
79	**pelargonic acid**	G	**944**	14	**486**	4	**372**	3	**744**	209	**778**	73	**645**	82
		V	**640**	80	**546**	41	**1012**	73	**1294**	30	**713**	46	**2199**	158
		M	**297**	72	**1492**	43	**528**	194	**1400**	59	**495**	103	**435**	27
80	**palmitic acid**	G	**2687**	167	**3023**	74	**2515**	226	**2511**	47	**2177**	51	**2656**	160
		V	**2147**	234	**2764**	102	**2645**	64	**2558**	182	**2897**	185	**2912**	14
		M	**1830**	46	**3330**	212	**2999**	348	**3314**	546	**3581**	712	**2747**	60

Continued on next page - * G, Green pea; V*, veraison*; M, Mature

Continued

Table 1. Changes in the metabolome of grape berries from Alvarinho, Arinto and Padeiro de Basto from North and South Portugal as assessed by GC-TOF-MS.

			Secondary metabolism											
	Metabolite	**Stage***	**Alvarinho**				**Arinto**				**Padeiro de Basto**			
			North		South		North		South		North		South	
			mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
81	**ethanolamine**	G	**1272**	1215	**1409**	78	**1812**	7	**1799**	535	**2270**	100	**1002**	22
		V	**466**	472	**401**	16	**544**	142	**464**	83	**335**	55	**386**	46
		M	**342**	22	**298**	43	**255**	39	**222**	46	**245**	48	**348**	48
82	**epicatechin**	G	**1033**	10	**1054**	53	**1229**	60	**621**	128	**1770**	55	**557**	19
		V	**10669**	218	**8753**	35	**18150**	93	**32438**	1444	**5297**	46	**6176**	100
		M	**2296**	62	**1431**	66	**4036**	549	**2208**	65	**1667**	146	**674**	83
83	**catechin**	G	**27779**	1540	**36209**	42	**35653**	305	**23711**	807	**44757**	806	**18740**	73
		V	**25011**	810	**22951**	23	**16303**	81	**30378**	838	**4782**	59	**8388**	57
		M	**5360**	30	**3821**	50	**2621**	416	**2237**	75	**1236**	123	**599**	27
84	**caffeic acid**	G	**270**	57	**492**	33	**278**	32	**454**	113	**523**	74	**431**	35
		V	**207**	10	**161**	28	**230**	70	**230**	61	**217**	20	**178**	12
		M	**175**	21	**152**	19	**152**	40	**152**	29	**239**	84	**145**	16

7.2 *VvHT1* coding region

>(gi|4138723:1-133, 237-559, 1424-2053, 2140-2613) *Vitis vinifera* hexose transporter 1 gene,

ATGCCGGCTGTCGGAGGCTTTGATAAGGGTACCGGGAAGGCCTATCCCGGTAACCTTACTCCTTACGT
GACTGTGACATGTGTTGTTGCAGCCATGGGTGGTTTGATCTTTGGTTACGATATTGGAATTTCTGGTG
GGGTCACGTCCATGGCTCCGTTCTTGCAGAAGTTCTTCCCTTCTGTGTACCGGAAGGAGGCTTTGGAC
AAGTCCACGAATCAGTACTGTAAGTTTGATAGTGAGACACTGACGTTGTTCACATCGTCGCTTTATCT
GGCTGCTCTTCTCTCGTCGCTGGTGGCCGCGACGGTGACCCGAAAGTTCGGGAGAAAGCTGTCAATGC
TATTCGGAGGACTGCTCTTTTGTGCCGGTGCCATCATCAATGGCGCTGCTAAAGCTGTTTGGATGTTG
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TGAGATGGCTCCATACAAATACAGAGGAGCCCTCAACATTGGCTTCCAATTATCCATCACAATTGGTA
TTCTTGTGGCCAATATATTGAACTACTTCTTTGCAAAGATCAAGGGGGGTTGGGGATGGAGGTTGAGC
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CTCCATGATCGAGCGTGGGCAGCACGAGGGAGCGAAAACAAAACTGAGAAGAATCCGGGGTGTCGATG
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CGTTAACATGTTTTTCACATTCATCATAGCCCAAATCTTCTTAAATATGCTGTGTCACATGAAGTTTG
GTTTGTTCCTCTTCTTTGCCTTCTTTGTGGTGGTGATGTCCTTCTTCATTTACTTCTTCTTGCCTGAG
ACCAAGGGCATCCCAATTGAAGAGATGGCTGAAGTATGGAAAAGTCACTGGTTCTGGTCCCGGTATGT
CAACGATGGTTCTTACAGCGGCGTCGAACTGGTCAAGGAAAACTACCCTGTTAAGAATGTATGA

7.3 *VvHT6* coding region

>gi|61613104|gb|AY861386.1| *Vitis vinifera* hexose transporter 6 (HT6) mRNA, complete cds

GTGGCATGAAATTCTCCAGAACTCAACGGTTACGCTTGATTCTTATTTGTCTAGTTCTCCAGATTCGC
CCTCAACACACTCTCTTTCTCGAGCCCCCTTCCCTGATTTTGGTGTCTTCAAAGAGGTTAGGCCAAGA
TTGGGGGTAAAGTTGATTGCTGAGAAACCATGAACGGAGCTGTGCTAGTGGCTATTGCAGCCGCGATT
GGTAACTTTCTGCAAGGATGGGATAATGCTACTATTGCAGGGGCTATTGTTTACATAAAGAAGGAGCT
TGATTTGGAGAGCACTGTAGAAGGGCTTGTTGTGGCTATGTCCCTCATTGGAGCCACATTGGTCACAA
CATGCTCAGGAGCCATATCGGATTGGATTGGTCGGCGTCCAATGTTAATAGTCTCATCAATGCTTTAT
TTTATCAGTGGTTTGATAATGTTATGGTCACCAAATGTCTATGTTCTACTTATAGCTAGGTTACTTGA
TGGATTTGGAATTGGTCTAGCAGTTACTCTTGTTCCCATTTATATATCTGAGACTGCCCCAGCAGATA
TAAGGGGATCATTAAATACTCTTCCACAATTCACTGGTTCTGGTGGAATGTTTTTATCATACTGCATG
GTTTTTGGGATGTCATTGTTAAGCTCACCAAGCTGGAGGTTAATGCTTGGGATTCTTTCAATTCCATC
CCTTCTTTATTTTACTTTGACAGTATTTTACTTGCCCGAATCCCCACGGTGGCTTGTAAGTAAAGGAA
GGATGGTTGAGGCTAAAAAGGTTCTTCAAAGATTACGTGGCAGGGAAGATGTTTCAGCTGAAATGGCT
TTGCTGGTTGAGGGTCTTGGGATTGGGGGTGAAACATCCATAGAAGAGTACATAATAGGGCCAACTGG
TGAGCTTACTGAAGATCAGGATCCAGATGCTGTGAAAGACCAAATCAAGTTATATGGACCTGAAGCTG
GCCTCTCCTGGGTTGCCAAACCTGTTCCTGGTGGACAGAGTACCCTTAGTCTTGTGCCTCGCCAAGGA
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GCCCCCTGAAACGGGAAGTATGCGAAGCATGCTTTTTCCCAATTTTGGCAGCATGTTCAGTACAGCAG
ATCCTCAGATTAAAACTGAACAGTGGGATGAAGAGAGCCTACAGCAGGAAGGTGAAGACTACGCATCA
GATGGTGGGGGAGACTCTGACCATGATTTGCAAAGTCCATTAATATCTCGCCAGACAAGCAGCATGGA
AAAGGACATGGTGCCCCCTCCTTCCCATAGCAGCATAATGAGCATGAGGCGTCATAGCAGTCTGATGC
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TCTGAGAGAGAAGGTGAAGATGGAAAGAAGGAAGGAGGATTTAAAAGGATTTATTTGCATGAGGAGGG
AGTCCCCGGATCCCGACGTGGGTCTCTTGTTTCACTTCCTGGTGGAGATGTTCCTGCAGAGGGCGATT
ATATCCAGGCTGCTGCTCTAGTGAGTCAGCCTGCTCTTTACTCTAAGGAGCTTATGGATCAGGATCCG
GTTGGACCTGCAATGGTTCACCCAGCTGAAACTGCTTCAAGAGGGCCA
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AAGTTCTTCTCGAGAGCTTGGGGCTTGGTACGGAGTCTGCATCGTTCCTTATCAGTGCATTCACAACC
CTCTTGATGCTTCCTAGTATAGTCGTCGCCATGAAGCTCATGGATATCGTTGGCAGAAGGCGCATGCT
GCTCACTACAATTCCTGTGCTGATCGTCACACTTCTCGTCCTAGTCATCGGCGACCTTGTGACCACGA
CCACAGTGATCCATGCAGCGATCTCTACTGCATGTGTTATCATCTATTTCTGCTGTTTTGTGACGGCG
TATGGACCAATCCCTAACATCCTCTGCTCTGAGATCTTCCCCACAAGGGTCCGAGGCCTGTGCATTGC
CATATGCGCTCTAGTTTACTGGATTGGGGACATCATCGTCACCTATACGTTACCGGTGATGCTGACTT
CAATCGGGTTGACGGGTATCTTTGGAATTTATGCAGTTGTGTGTGTGATTTCTTGGGTGTTTGTGTTC
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CCTTCTTCC