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Effect of irrigation on berry and skin cell wall composition in the grape varieties Touriga Nacional and Trincadeira

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Abbreviations

AG-I	Type I Arabinogalactan
AG-II	Type II Arabinogalactan
AGP	Arabinogalactan Protein
CDTA	Trans-1,2-Cyclohexanediaminetetraacetic Acid
CWM	Cell Wall Material
EDTA	Ethylenediaminetetraacetic acid
Etc	Crop Evapotranspiration
F3’5’H	Flavanoid 3’,5’-hydroxylase
FI	Fully-Irrigated Treatment
Gal*p*A	Galactopyranosyluronic
HG	Homogalacturonan pectin
KOH	Potassium Hydroxide
NI	Non Irrigated Treatment
PRP	Proline Rich Proteins
RG-I	Type I Rhamnogalacturonan
RG-II	Type II Rhamnogalacturonan
WAK	Wall-Associated Kinase proteins

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Abstract

The main objective of this study was to determine how water availability effected the composition of both the grape berry and the skin cell wall of two Portuguese grape varieties, Touriga Nacional and Trincadeira. Different vines situated in the Centro Experimental de Pegões, 70 Km East from Lisbon, Portugal, were subjected to Non-irrigated (NI, no water applied) and Fully-irrigated (FI, 100% of evapotranspiration rate) treatments throughout the growing season for each variety.

Berries were harvested and sampled on 20th September 2010 from the four plots and the yield and quality parameters were tested. Differences between the treatments were noted with grapes from irrigated plants showing significantly higher berry weight, and volume. Whereas the non-irrigated berries showed higher levels of anthocyanin content, colour intensity, total phenolics and total acidity, than the fully irrigated. There were also differences noted between varieties in several parameters, mainly with Touriga Nacional showing a higher content in the phenolic compounds.

Slight differences were found in the cell wall composition of the berries, with fully irrigated treatments showing higher levels of cellulose. There was a decrease in total sugar content, and total uronic acid content within fully irrigated berries for both varieties. In addition, Touriga Nacional showed an increase in neutral sugars under the fully irrigated treatment, where as irrigations showed to decrease the level of neutral sugars for Trincadeira.

It is possible that the extractability is correlated to the decrease in total sugar and pectins that may aide the release of the cell wall bound phenolic compounds to the must.

Keywords: Touriga Nacional, Trincadeira, Irrigation, Cell Wall, Polysaccharides, Extractability Index

Extended Abstract

Vitis vinifera has proven to be a very versatile plant, by being successfully grown and survive as produce in a range of different climates and conditions. Ranging from extreme cold winter temperatures to hot deserts. However, climate change is fast becoming a concern for many farmers around the World, with higher average temperatures and drier summers. As a result, farmers are aware that this could mean change in the behaviour in the plant, and possibly even quality in the fruit produced. In many regions, irrigation has been implemented to combat the harsh conditions and increase yield to a sustainable level.

Portugal is a country that is host to a range of climates, with generally cooler wetter grape growing regions in the north (e.g. Minho) and warmer, drier regions in the south (e.g. Alentejo). As a consequence, some of the regions further south have come to rely on irrigation as a water source for the crop. In addition, Portugal plays host to the largest pool of indigenous grape varieties due to its traditional heritage of winemaking. Much of Portugal's success in the global wine market has been accredited to the red wines produced. As a consequence, two well know red Portuguese varieties were chosen to be the focus of this study, Trincadeira and Touriga Nacional.

Phenolic compounds, for example tannins and anthocyanins, that are viewed to be beneficial to wine quality are generally stored in the skin of the berry and are transferred to the must during the maceration period, where the must remains in contact with the skins for a period of time. However dark varieties with high anthocyanin levels, a molecule associated with colour, do not necessarily produce wines deep in colour, as the cell wall acts as a biological barrier. As a result, the aim of this study is to determine if there is any association between the composition of grapes grown under two different conditions, with one fully irrigated and the other non–irrigated, and whether the water availability has an effect on the cell wall composition, as well as identifying any varietal difference.

A block of non-irrigated and fully irrigated of each Touriga Nacional and Trincadeira, were studied in a vineyard situated 70 kilometres east of Lisbon, Portugal, in the Alto Alentejo, at the Centro Experimental de Pegões (38°39'1"N 8°38'42"W). The vines from the non-irrigated blocks were confirmed to be under water stress with water

potentials of -0.825mPa and -0.625mPa for Touriga Nacional and Trincadeira respectively, whereas the fully irrigated was not. At harvest, on 20th September 2010, for all blocks, 100 berries were sampled.

The berries were assessed for berry composition, and there proved to be significant differences between treatments for berry weight, berry volume, Brix, pH, total acidity, colour intensity, anthocyanin content and total phenolic. In terms of irrigation, there was a reduction in potential wine quality with the presence of water, with a decrease in total phenolic compounds, colour intensity and anthocyanin content. Grapes from fully irrigated vines were also shown to have a larger berry weight and volume. There were also significant differences between varieties, with Touriga Nacional having higher phenolic content with a smaller berry.

In terms of cell wall composition, vines that were fully-irrigated had significantly higher amounts of cellulose than the non-irrigated, with Trincadeira showing higher cellulose levels for both treatments. In contrast, the general trend for total sugars in the cell wall after isolation was to decrease with full irrigation for both varieties under the three fractions (CDTA, 0.1KOH & 6M KOH). The only exception was the increase in total sugars extracted with irrigation with Touriga Nacional. The pattern was reflected with a decrease of uronic acid content with irrigation within the CDTA and 0.1M KOH fractions and no significant differences noted for 6M, which suggested and water availability had no effect on the more tightly bound pectins. There was also an increase in neutral sugars extracted in both the 0.1M KOH and 6M KOH fractions for Touriga Nacional under full irrigation, whilst only a decrease was found in the 6M KOH fraction for Trincadeira. Within the polysaccharides extracted, there was little difference between the varieties, suggesting that cell wall composition is fairly standardised between the varieties. In addition, total cell wall protein content did not vary between the varieties or treatment.

Without further analysis of identification of individual sugars and molecules present, it is impossible to determine or correlate the differences in extractability. It is possible that the decrease in pectins found with irrigation, through the uronic content, may play a role in the increase in extractability. As in previous studies it has been noted that extractability increased through possible degradation of pectins, especially AG-I, a sidechain associated with the softening of the skin *postveraison*.

Keywords: Touriga Nacional, Trincadeira, Irrigation, Cell Wall, Polysaccharides, Extractability Index

I. Introduction

Grape production accounted for 67.95 million tonnes in 2010 (OIV- International Organisation of Vine and Grape) much of which was used for a range of products, including fresh table grapes, raisins and jams. However about 80% of grapes cultivated are used in winemaking (Kammer *et al.,* 2004). A lot of research has been focused on the optimising the quality of wine by understanding the behaviour of different varieties under different vineyard conditions and management.

Touriga Nacional is a native variety from Portugal that is known for it's perceived high quality red wine and port wine. Even though Portugal still have the largest area of planted Touriga Nacional (Nel, 2009), the potential wine quality the variety can bring has led to an increase in plantings in other countries such as South Africa, where 87 hectares have been planted in 2008 (Van Wyk & Le Roux, 2009), as well as plantings in California, United States of America, Argentina, and Australia (Higgs, 2009).

As a variety, Touriga Nacional is naturally very vigorous and is therefore best when grown in low potential soils. It is in these soils, where Touriga Nacional has enhanced its reputation as a variety to produce high quality wines. One of these regions is the Douro, situated in the north of Portugal, where it is home to the largest area planted of the variety. The Douro's schistous soil, with its low water holding capacity, low fertility and nutrients restrains the natural vigour of the variety. It is also under these restricted growing conditions, that Touriga Nacional produces smaller clusters, with smaller, oval shaped black berries and display a lower pH value with a high total acidity at harvest (Oliveira *et al.,* 2006, Shellie, 2007). In addition the variety shows, a high phenolic content in both the wine (Andrade *et al.,* 1998) and in grapes at harvest, which is ideal for the production of wines produced for ageing, like vintage ports (Mateus et al., 2001). It also considered that Touriga Nacional has high aromatic properties, with favourable aromas associated with red fruit (cherry and raspberry), jammy black fruits and especially floral notes (violet and rose) due to the high terpenol (Guedes de Pinho *et al.,* 2007) and norisoprenoid content, especially β-ionone (Oliveira, 2006).

Trincadeira is another native Portuguese variety that is grown in all regions of the country, however new plantings are on the decrease due to the susceptibility of infection, mainly by *Botrytis cinerea* and *Plasmopara viticola* (Fortes *et al.,* 2011). Wines from this variety tend to have a good structure and possess good aromas through their high levels of carotenoids (Oliveira *et al.,* 2006). Trincadeira is often used as a variety blended in with others due to its ability to maintain a high acidity and low pH at harvest with a reasonable colour and phenol levels (Abade & Guerra, 2008).

In terms of red wine making, much of the sensorial characteristics and quality are due to the phenolic compounds located in the grapes. These characteristics include the colour, tannic structure and aromas and their precursors. The phenolics are generally located in the skins of the berries, and the content of the compounds differ with the variety (Pinelo *et al.,* 2005; Rodríguez Montealegre et al., 2006) and even between Touriga *Nacional* and Trincadeira (Abade & Guerra, 2008). The transferring of the phenolic compounds to the wine occurs during the maceration stage of winemaking, where the skins (along with seeds and sometimes stems) of the crushed berries remain in contact with the must. The rate at which the compounds transfer to the must is very much dependent on the winemaking technique (Salas *et al.,* 2003).

II. Literature Background

II.1 Berry Anatomy

The development of the vine up to the point of perceived maturation of the grape is vital in determining the behaviour of the berries in general, and the differences between varieties. In terms of winemaking, it is essential to better understand the processes undergone by the vine and the berry in order to produce the desired product. As behaviours of the vine under different conditions will determine factors such as vineyard management, harvest date and winemaking techniques.

The anatomy of the berry is reasonably universal among the varieties with some differences, mainly between shapes of the berry and the number and shapes of seeds (generally between 1-4).

The skin, or *exocarp*, of the berry is found on the outside of the berry and constitutes between 5% and 18% of the total mature berry weight. The exocarp is comprised of different cell layers, with the most extreme outer layer being the cuticle with varying thickness. The cuticle consists of hydrophobic lipid waxes (see figure 1.1), as well as hydroxylated fatty acids called cutin, that act as a protective layer to the plant tissue from different forms of potential injury due to wind, physical abrasion, frost, sun radiation and infections from pathogens, fungi and insects (Rosenquist & Morrison, 1988) and limit water loss (Rogiers *et al., 2004)*. The structure of the wax changes through the development of the berry, degrading slowly as the berry matures. Epidermal cells (6.5 to 10μm in width) and hypodermal cells (10 to 12 cell layers)

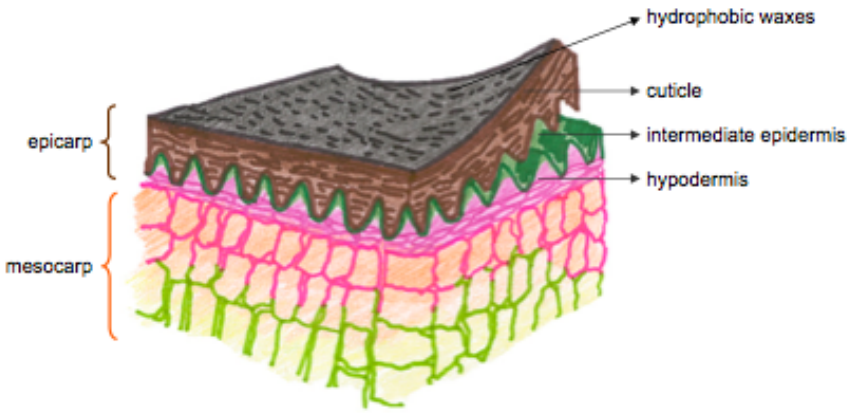

Figure 2.1: The different layers of the grape berry skin (taken from Pinelo et al., 2006)

also make up the exocarp (Alleweldt *et al.,* 1981). The hypodermal tissues are rich in polyphenols, especially in their vacuoles (Barceló *et al.,* 1994; Lecas & Brillouet, 1994).

The pulp, or *mesocarp*, is composed of a layer of 25-30 parenchyma cells. The cell walls undergo modification throughout development and soften during and after veraison (Coombe & Bishop, 1980) through the hydrolysing of cellulose and hemicellulose in the wall (Yukushiji et al., 2001). The decrease in cellulose is compensated by the insertion of glycoproteins to reinforce the cell walls throughout the softening process (Davies *et al.,* 1999). At maturation, the vacuoles within the parenchyma cells make up to 90% of the volume with organic acids and sugars. Deeper within the mesocarp, is the *endocarp*. The seeds are contained within the endocarp and have a substantial phenolic content, between 5 to 8% of the total seed weight (Shi *et al.,* 2003).

Within the mesocarp, the *vascular bundles* nourish the berry with some bundles situated in the within the endocarp, and an extensive network situated in the periphery of the berry. The bundles contain both a xylem and a phloem to transport nutrients to the mesocarp in both a symplastic and apoplastic manner.

II.2 Berry Development

Understanding the manner in which the berry develops over a season is a necessity in optimising quality, energy and cost in both the fruit and wine produced. Berry growth and volume follows that of a sigmoid curve, as there are two growth phases, the herbaceous growth phase and maturation, with a lag phase separating the two.

Immediately after fertilisation, the ovary begins to swell, and become rounded. The capillary walls are become rich in tannins and the peripheral bundles separate the hypodermis and the parenchyma. It is in the initial days after the swelling of the ovary when the rate of cell division it as it's highest. This first phase of growth is split into three stages. Within the first five days after anthesis, there is a very high rate of mitosis, with little focus on cell enlargement. Between days five and 35, the rate of mitosis decreases significantly, and the induction of cell enlargement. After the day twenty, the number of cells within the berry is loosely fixed, with around 75% of the cell and DNA multiplication already having occurred (Ojeda *et al.,* 1999). The cell

enlargement is generally attributed to the uptake of water by the xylem in the vascular bundles. Very low levels of sugars are present in the berries, as those that are present are broken down by glycolytic enzymes into organic acids. This is a source for the biosynthesis and the accumulation of tartaric acid as well as the catabolism of vitamin C (DeBolt *et al.,* 2006). Malic acid also derives from the sugar glycolysis pathway or the carbonylation of pyruvic acid. Throughout this process there is also increase in tannin content within the seeds and the skins. The third stage occurs with the last week of stage 1, where cell multiplication ceases and the emphasis is on cell enlargement. This initial stage of berry development is very sensitive to environmental conditions. Temperature affects the rate of cell division and enlargement, with the optimum being between 20-25^{o}C, and those over 35^{o}C severely reducing the growth rate. Sunlight stimulates cell division, therefore affecting the berry size, with shaded conditions resulting in smaller berries. Water stress does affect the size of the berry at this early stage, but it does not seem to affect the rate of cell division (Ojeda *et al,* 1999).

Following the herbaceous growth phase, there is a lag phase, where the berry does not increase in size and undergoes preparatory measures for *veraison*. The duration of the lag phase is dependent on the cultivar and the conditions.

Veraison is the onset of the rapid second growth phase (Coombe, 1972) – grape maturation. Veraison is charaterised by the immediate softening of the berry, usually within 24 hours, and the change in skin colour for the red cultivars. The softening of the berry is associated with the loosening of the cell wall, firstly in the mesocarp and followed by the exocarp. Within the mesocarp, this allows an influx of sugar, in the form of sucrose, and water into the vacuoles via the phloem. The sucrose will be hydrolyzed to produce glucose and fructose present in the berry at maturity. This influx and cell wall softening allows further and rapid cell enlargement (Huang *et al,* 2005). Within the mesocarp, the levels of organic acids decrease, and as the pH and the level of sugars increase. Whereas in the exocarp, the level of chlorophyll declines, with the build up anthocyanins, tannin polymerisation and aroma compounds. The exocarp becomes stretched with the berry enlargement. This second growth phase continues until the berry reaches maturity, where the growth rate will decrease and eventually cease. After which the berry can shrink with overripeness with water loss within the fruit.

II.3 Berry Cell Wall Introduction

The cell wall is a complex macromolecular structure that provides the plant cell with a number of functions. The principal being mechanical and structural support and the determination of cell shape and control of cell growth, as well as providing protection against turgor pressure, pathogens and dehydration (Brett & Waldon, 1996). All cell walls show different heterogeneity depending on many factors including the organism and the tissue, but they are based around the same principals. Cell walls are a matrix of cross-linked polymers, microfibrils and other macromolecules (Goulao *et al., 2012*). Two adjourning cells will have two walls joined by the *middle lamella.* The cell walls play a vital role in the shape and strength of the plant cells, as a result of the interlinking complex matrix of polysaccharides (including cellulose, hemicellulose and pectins) and glycoproteins (including Aribinogalactan proteins and extensin) (Goulao *et al.,* 2012).

The primary cell wall structure for dicotyledonous species, such as *Vitis vinifera*, have Type-I cell walls, and approximately 90% made up of polysaccharides (Carpita & Gibeaut, 1993). Within the matrix there are long polysaccharides microfibrils of cellulose acting as the backbone of the cell wall, as it provides much of the strength and the framework in which all other components are positioned. These microfibrils are aligned in the parallel to resist any perpendicular tension from the two adjourning cells (Goulao *et al.,* 2012). The microfibrils are themselves composed of 40 cellulose chains bound together with hydrogen bonds. The cellulose chains consist of covalently bound unchained β-1,4-linked D-glucose molecules.

These polysaccharide microfibrils are bound through hydrogen bonds to other polysaccharides, called hemicelluloses. Xyloglucans are the most abundant form of hemicellulose in dicotyledonous plants, and account for 8-12% of total cell wall polysaccharide fraction in the mesocarp and exocarp. There have been eight oligosaccharide structures identified in the grape berry, with very similar distributions between the mesocarp and the exocarp (Doco *et al.,* 2003). The xyloglucans, like all hemicelluloses, provide further reinforcement by crosslinking the cellulose microfibrils, and the middle lamella. As a consequence, these networks of hemicellulose fibres provide further resistance to tension on the cell wall. The network of polyscaccharides contains very stable cellulose and hemicellulose fibres, and the matrix of how these polysaccharides are bound may affect the firmness of

the berry. The structure of the xyloglucans are similar to cellulose in that they share the same β-1,4-link, however, 75% of the glucose have a α-1,6-linked D-xylose residue (Thompson & Fry, 2000).

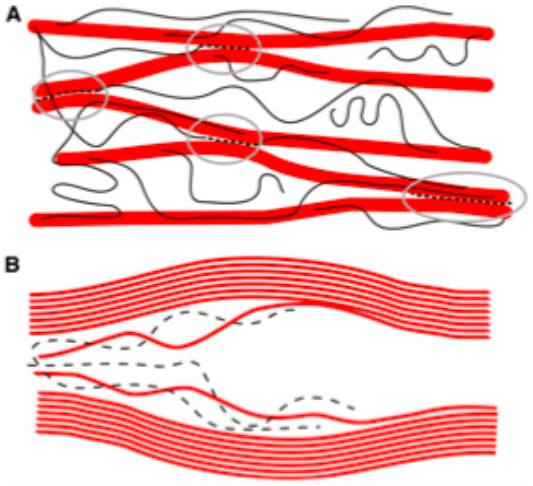

Figure 2.2: A simplified cartoon of the tethered network model of the primary cell wall. A. The xyloglucans (thins strands) are bound to the cellulose microfibrils (thick red strands) to reinforce the structure. The xylogucans represented by the broken line (highlighted in gray circles) are the load bearing regions, where two microfibrils are adjoined to the same xyloglucan. B. The hypothesised mechanism in which the intertwining xyloglucans are bound to the microfibrils with hydrogen bonds within the load bearing regions. (from Park & Cosgrove, 2012)

The mechanism of the xyloglucans within the macrostructure of the cell wall is of much debate. Some research suggests that the microfibrils are held into position by the xyloglucans, and that there are no interactions between the microfibrils themselves, thus giving rise to the tethered structure (Ordaz-Ortiz *et al.,* 2009). These xyloglucans are thought to bind, by hydrogen bonding, to exposed glucan chains of one of the microfibrils and bind to one another, reinforcing the strength of the structure of the cell wall. Or they can inter lock with other cellulose bound xyloglucans, having the same effect (Doco *et al.,* 2003). Therefore within this network model, the microfibrils are based to be 20-40nm apart from each other. The xyloglucans have three domains identified: strands accessible by xyloglucanase, the enzyme responsible for hydrolysising the xlyoglucans, and the strands include: free strands, loops and hypothetical tethers. The second domain being tightly bound to the microfibil that is not accessible by xyloglucanase and with the hydrogen bonds being degraded in potassium hydroxide (KOH). The final domain is only released upon complete wall digestion and thought to be situated inside or between the microfibrils (Park & Cosgrove, 2012).

However in a recent study, through a set of xyloglucanases with varying substrate specificities, several segments of xyloglucans were found to be inaccessible. This suggests that these regions of the same xyloglucans are intertwined and form a complex with possibly more than one microfibil (see *figure* 1.2; Park & Cosgrove, 2012). Other hemicelluloses have been identified in trace volumes including

mannans, glucomannans, galactomannans, galactoglucomannans and xylans (Lecas & Brillouet, 1994).

Pectins are third family of polysaccharides that are assembled in the intrinsic matrix of the cell wall, intertwined around the cellulose-hemicellulose network, as well as being the principal component of the middle lamella. The pectic polysaccharides contribute to the structure and functions, including regulating hydration and ion transport, of the cell wall by forming a hydrophilic gel. The gel also controls the permeability of the wall for enzymes in addition to providing support from compression. The biological role within the cell wall is determined by the ion balance, pH and surface charge, together with their complex structure and composition. Differences have been found between tissues of the berry with the exocarp appearing to have three fold more pectic fractions in the cell walls than in the mesocarp (Vidal *et al.,* 2001).

The principal pectins are Homogalacturonan (HG) and are present in higher levels in grapes than in other fruit and berries, accounting for 65% of the pectic fraction. HGs consist of α-1,4-linked galactopyranosyluronic acid (Gal*p*A) backbone with more than 72-100 residues (Thibault *et al.,* 1993) attached to glucose and xylose. The Gal*p*A, when not methyesterified at the C-6 carboxyl group, carries a negative charge. Ten or more unmethylesterified residues can form calcium bridges with other residues with the ionic bonding with calcium ions (Ca^{2+}), thus further stabilising the gel. However the Gal*p*A methylestification is very tissue specific and tightly regulated, and when esterified, the residue will carry a neutral charge and no longer able to form ionic bonds with the calcium ions.

Rhamnogalacturonan I (RG-I), 10 % of the pectic fraction, share a Gal*p*A backbone and α-1,2-linked rhamnose residues, attached to α-1,5-L-arabinans, β-1,4-D-galactans and arabinogalactan sidechains, type I (AG-I) and type II (AG-II) (Saulnier *et al.,* 1988). Arabinose and galactose are normally the terminal residue and the content is dependent on the development of the berry.

Rhamnogalacturonan II (RG-II) (Saulnier & Thibault, 1987; Nunan *et al.,* 1997) with a α-1,4-linked galactopyranosyluronic acid backbone with 4 different side chains linked to 12 other sugars including glucose, rhamnose, arabinose and galactose, with the

potential of forming borate diester covalent links with each other with the presence of calcium.

The berries are found to be abundant in AG-II (Saulnier & Brillouet, 1989), whereas they are low in RG-II polysaccharides in both the exocarp and mesocarp (Lecas & Brillouet, 1994). This is of importance as RG-II chains are found in wine, and can even affect a haze formation with the binding to other heavy metals (Szpunar *et al.,* 1998) as well as arabinogalactan-proteins.

In terms of the grape skin, cell wall weight is compromised 30% of neutral sugars (including cellulose, xyloglucans, arabinans and xylans) and 20% pectins, with under 5% of structural proteins at maturity (Lecas & Brillouet, 1994).

The primary cell wall is also composed of structural proteins and protein rods to support the long polysaccharide chains present. Extensins are one class of protein rods that are rich in hydroxyproline that can link to the polysaccharides within the cellulose microfibrils (Mort & Lamport, 1977) and act as support. Other proteins include proline rich proteins (PRP), glycine rich proteins (GRP), solanaceous lectins and arabinogalactan proteins (AGP) involved in cell adhesion (Majewska-Sawka & Nothnagel, 2000).

Phenolic compounds are also present within the cell wall of the exocarp, and they are of grave importance to the winemaker due to their influence on the character and perceived quality of the wine. This group encompasses as large family of compounds that affect the sensorial attributes of the wine, including the astringency, mouthfeel, structure, ability for a wine to age and the colour for red wines. In addition, there has been research to suggest that the phenolics may provide antioxidant properties (Lurton, 2003) and potential health benefits (Damianaki *et al.,* 2000). The phenolics are localised to the stem, berry seeds and mostly in the berry skin, with the total content within the skin ranging from 285 to 550mg of phenol/kg of grape skin (Pinelo *et al.,* 2005). There are a large number of different factors that affect the phenolic levels within the grape berry including, the variety, climatic temperature, sun exposure, soil type and water availability. The phenolic family can be catagorised into two groups: (1) flavanoids (flavanols, anthocyanins and proanthocyanidins) and (2) non flavoids (predominantly phenolic acids).

The flavanoids are based on the similar structural make up (see figure 1.3) called the *flavone*. There are two benzoic rings, ring A and B, with an unsaturated cationic oxygenated heterocycle, ring C, attached to an adjacent benzene ring, ring A.

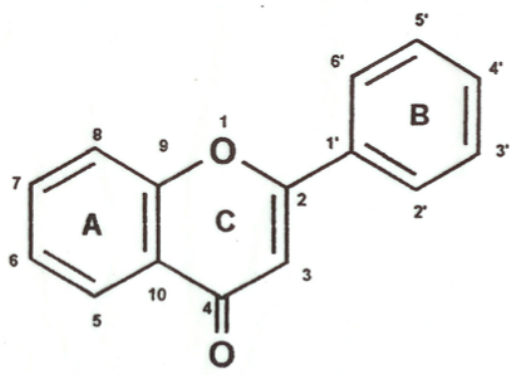

Figure 2.3: A flavone, which is the backbone of flavanoid compounds, shown with two benzene rings (A&B), and an unsaturated cationic oxygenated heterocycle (C).

It is known that red wine can only be made from red varieties (although some white varieties are blended into red wines, but only in small proportions), and it is the extraction of the anthocyanins from the grape skins to the must from crushed berries during the maceration process that provides the red, violet colour present in the wine (Salas *et al.,* 2003). These are water-soluble flavanoid derivatives, with six commonly occurring mono anthocyanidins occurring in the grape berry, (Delpinidin, Cyanidin, Petunidin, Peonidin and Malvidin). These are more often glycosylated or acylated at the C3 atom in the unsaturated cationic oxygenated heterocycle. The chemical environments in which the anthocyanins are in, greatly affect the structure and their colourisation. The quantities present in the must differs greatly with variety (Romero-Cascales *et al*.,2005).

The phenolic acids are mainly benzoic acids and cinnamic acids. Little is known about their role within winemaking, however from an organoleptic view, they are shown to be odourless and colourless.

Within the cell, the phenolic family can be found in a number of organelles, including the vacuole, cytoplasm, nucleus and the cell wall. Much of the research has been focused on the presence of anthocyanins within the vacuole of flowers, however some studies do suggest that non-acylated and acylated anthocyanins have been located within the grape berry skin vacuoles (Conn *et al.,* 2003). Flower research has suggested that these are thought to be free in solution, and also forming complexes with proteins within the vacuole membrane (Markham *et al.,* 2000). Additionally, the flavanoids have been shown to be expelled from the vacuole and found in the cytoplasm (Markham *et al.,* 2001). Moreover, sufficient levels of catechin, epicatechin

and proanthocyanidins have been located in the cell nuclei of different tree species (Feucht *et al.,* 2004), however their role within the nuclei is unknown.

Grape tannins, like anthocyanins, greatly affect the composition and perceived quality of a wine by affecting the sensorial side attributes in terms of mouthfeel, possible bitterness as well as stabilisation of the wine with time and therefore used as an indicator of the potential ageing ability of the wine. The tannins are polymerised 3-flavanols with varying polymerisation degree depending on the conditions, variety, and berry tissue; with the skin hosting the higher mean polymerisation degree, averaging ~28 (Souquet *et al.,*1996), higher than that found in the skins, stems and pulp. Within the grape skin, the main 3-flavanol units are catechin, epicatechin and epicatechin gallate. Grape cell wall also has extractable proanthocyanidins in suspension (Nunan *et al*.,1998; Hazak *et al.,* 2005; Bindon *et al.,* 2010). However much of the studies, have been carried out on apple cell walls. There have been two mechanisms hypothesised in which the proanthocynandins bind to the cell wall.

The first mechanism is through non-covalent bonding, mainly hydrogen bonding between the oxygen atoms of the crosslinking ether bonds of sugars present in the cell wall polysaccharides and the hydroxyl group of the phenols (Le Bourvellec, *et al.,* 2005). Further studies on apples suggested that the proathocyanidins had different affinities to different polysaccharides, by having the greatest affinity with pectins, then xyloglucans and lastly cellulose (Renard *et al.,* 2001). It was also found that the affinity also increased with the polymerisation degree of the proanthocyanidins and the portion of (+)-catechin.

The second mechanism is through hydrophobic interactions as some of the polysaccharides present can form secondary structures (for example gels) with hydrophibic properties that can encapsulate compounds such as flavanoids (Le Bourvellec *et al.,* 2005). These hydrophobic gels will hold the phenolic compounds within the matrix of the cell wall. Although these represent a relatively small portion of the total phenolics present in the cell, it is useful to understand the molecular structure of the compounds and their solubilisation.

Despite much of the research being focused on apple cell walls, many principals would apply to *Vitis vinifera.* However it should be considered that phenolic content is also present in the seeds and stems, as well as trace levels found within the pulp.

These phenolic compounds within the different tissues have lower mean polymerisation degree, as well as being localised purely in the vascular tissue and the cytoplasm. These factors should be considered both in terms or research and winemaking as harsh extraction of these lower polymerised proanthocyanidins, especially from the stems and seeds, will affect the bitterness and mouthfeel of the wine produced.

II.4 Modification of Berry Cell Wall Along Growth and Ripening

Softening of fruit is often associated with ripeness, and it is widely recognized that this is due to modifications within the pulp cell wall. During berry development, there are a number of modifications occurring within the cell walls of different tissues. During the first growth phase of development, the emphasis is on berry enlargement in all of the tissues, whereas in the transition period between veraison and the second growth phase, only the exocarp expands. Lastly, during the second growth phase, only the mesocarp undergoes cell expansion. The function of the cell wall is to maintain the structure, shape and size of the cell under high turgid pressure, as water passes through the membrane with osmotic pressure. Therefore the cell can only enlarge with the enlargement of the cell wall.

Most of the cell wall modification of the skin occurs post veraison, as the skin begins to soften. The softening of the tissue and the cell walls in question leads to further cellular growth of the mesocarp with the influx of water and sugars typically seen in during the second growth phase. A thinning of the cell wall material (CWM) may be seen throughout the maturation of the berry, although there is a debate whether total cell wall decreases during this period with a decrease being observed in some varieties (Monastrell and Cabernet Sauvignon) (Ortega-Regules *et al.,* 2008) and no change seen in others (Syrah) (Vicens *et al.,* 2009). A degradation of the middle lamella in the hypodermis cells has also been noticed to aide with the cell expansion within the mesocarp (Huang *et al.,* 2005). Although there have been decreases of CWM witnessed in the mesocarp of the Gordo variety (Nunan *et al.,* 1998) as well as a decrease during the ripening of the cherry (Batisse *et al.,* 1996) and strawberry (Rosli & Civello, 2004).

Despite the drastic softening of the skin, only moderate changes occur within the cell wall composition. Over the period of ripening, and as the berry reaches maturity, the

content of water-soluble sugar fraction roughly doubles within the berry skin cell walls from 3 to 8% of total sugar content (Vicens *et al.,* 2009). This is in accordance with changes in the mesocarp, however the levels of the water-soluble fractions are higher with an increase of 10 to 23% (Nunan *et al.,* 1998). In addition, the contents of insoluble pectins also decrease (Huang *et al.,* 2005). This is in parallel with the decrease in insoluble galactose levels within the cell wall (Ortega-Regules *et al.,* 2008; Vicens *et al.,* 2009), with this mainly being attributed to the degradation and solubilisation of AG-I sidechains as soluble arabinose levels increase whereas rhamnose levels remain constant. Enzymatic activity also suggests the solubilisation of the sidechains, with α-Galactosidase and β-Galactosidase activities increasing dramatically increasing as the berry softens (Nunan *et al.,* 2001). Although this was in the mesocarp, similar activities would be expected in the skin.

Moreover, this is in conjunction with a decrease in the calcium bridge bound pectins, also attributing to the loosening of the cell wall (Huang *et al.,* 2005). The calcium ions may be translocated into the cytoplasm of the cells as they are required for the cell to function. This is possibly due to localised pH decreases within the cell wall, with the acidification being the cause of the cleaving of the bridges. Additionally there is also an increase in the pectin methylesterase activity in catalyzing the hydrolysis of the methylester groups in the pectins of the cell wall (Nunan *et al.,* 2001). Both mechanisms would lead to an increase in the loosening of the cell wall with weaker interactions between the polysaccharides. However the rate of decrease of methyl and acetyl-esterification is variety specific, with dramatic changes witnessed in Cabernet Sauvignon, Monastrell and Merlot, with little change in Syrah (Ortega-Regules *et al.,* 2008).

Studies have shown conflicting results in terms of cellulose degradation throughout ripening. Several studies have shown that cellulose levels did not decrease throughout maturity (Ortega-Regules *et al.,* 2008; Nunan *et al.,* 1998), whereas others have shown a decrease in cellulose and hemicellulose levels in the skin (Ishimaru & Kobayashi, 2002) and in the pulp (Yakushiji *et al.,* 2001), suggesting a different mechanism for different tissues. Additionally, acidification also cleaves the hydrogen bonds binding the xyloglucans to the cellulose microfibrils (Huang *et al.,* 2005). This could suggest that the xylcoglucans are exposed to enzymatic activity, or more likely that it is an unwinding of the rigid matrix structure, resulting in more fluid model.

With the partial degradation of polysaccharide material, the cell wall compensates with the incorporation of proteins throughout the maturation period. These proteins are found to be rich in hydroxyproline and thought to be the glycoprotein family, extensins. The increase in hydroxyproline amino acids after veraison suggests the extensins are synthesised and incorporated into the cell wall at this period (Huang *et al.,* 2005). In conjunction with the increase in peroxidase activity in the cell wall of the four outer most cell layers of the skin, including the epidermis and subepidermis. Peroxidase is an enzyme associated with the irreversible phenolic cross-linkage between polysaccharides and proteins (Calderon *et al.,* 1993). As a result the incorporated proteins most likely reinforce the cell wall, and limit the degradation of the polysaccharides as well as preserving the integrity of the cell wall of the skin in order to act as a protective barrier against infection and water loss. The incorporation maybe an important difference between the softening of the cell walls within the pulp and the skin.

The evolution of the phenolics located within the skin cell wall is scarce as most of the phenolics are found within the vacuole. Although as stated, proanthocyanidins are present within the cell wall (Nunan *et al*.,1998; Hazak *et al.,* 2005; Bindon *et al.,* 2010), by binding to pectins. However some research does suggest that there is little or a very slight decrease in total phenolic content covalently bound to the cell walls over the ripening period (Ortega-Regules *et al.,* 2008).

II.5 Water Availability on Berry Composition

Many environmental factors, including water availability, affect the composition and characteristics of the grape berry. This in turn affects the grape quality in terms of phenolics for winemaking. With water being one of the most valued resource of the planet, it is vital to understand the impact and affect it has on grape development, therefore there has been extensive research on the effect of water on the behaviour of the vine and the quality of grapes produced.

The grapevine has the resources to survive and thrive in semi-arid climate, such as the Mediterranean, with physiological drought mechanisms with a efficient stomata control for transpiration paired with extensive root systems. However in some wine producing regions, temperatures and drought maybe excessive and irrigation is

needed for just the vine to survive. However in terms of grape quality, irrigation is a highly debated area as moderate water supply can increase the yield and even the grape quality (Hepner & Bravdo, 1985). On the other hand, irrigation has also been shown to have a detrimental effect on grape quality, especially in the sugar content and colour through to anthocyanin content (Dokoozlian & Kliewer, 1996), due to increased canopy and vegetative growth, which can lead to further problems including fungal infection.

Water deficit between anthesis and maturity leads to a decrease in berry size, in terms of both berry weight and volume (Sojo *et al.,* 2012). However the mechanism in which this occurs is dependent on when the deficit is applied. Water stress during the stage I of growth, pre-veraison, results in smaller grapes in size (Ojeda *et al.,* 2001) and is irreversible, as restoration of the full quota of water later in the cycle does not restore them to their size when not under stress (Poni *et al.,* 1994). However this is not necessarily detrimental in wine quality, as this leads to a smaller skin to pulp ratio, which in turn will potentially increase the phenolics in the wine for red wines (Roby *et al.,* 2004) and aromas for white wine.

Post-veraison leads to an increase in phenolics in the mesocarp as well, but this is probably an indirect action to the water stress with the decrease in sugars in the grape from the decrease in photosynthesis. Moreover the exocarp is deemed to be thicker with smaller berries, and this has been associated with the decrease of activity in pectin methylesterase, one of the enzymes accredited for the loosening of the cell wall (Deytieux-Belleau *et al.,* 2008).

Deficit irrigation is also known to greatly affect the accumulation of the polyphenols within the exocarp and the seeds. As a result regulating the water stress of the vine is a powerful tool in managing the levels of these compounds to improve the final wine quality. Water deficit increases the anthocyanin in the grape skin (Matthews & Anderson, 1988; Intrigiolo & Castel, 2010) as well as causing smaller berries. The increase in anthocyanin content was observed when the water deficit was applied in both pre and post veraison for Cabernet Sauvignon (Castellarin *et al.,* 2007) and only post veraison for Syrah (Ojeda *et al.,* 2002). Therefore the water status must be having an effect directly, or indirectly through the vegetative growth, on the flavanoid pathway. In some cases, vines under moderate stress have shown there to be an increase in the extractability of the anthocyanins into the wine, than those under full irrigation (Koundouras *et al.,* 2009).

II.6 Aims of Study

The composition and the structure of the grape berry have implications on winemaking and the technologies used. In a competitive business world, a lot of research is aimed at optimising potential wine quality both in the vineyard and in the cellar. In the production of red wines, a lot of focus is aimed at the extraction of phenolics that will be beneficial to the wine quality, or stop extraction of undesired phenolics from grapes with a short ripening season.

From previous experience, a grape that contains high anthocyanin content does not necessarily produce must that is rich in colour. As a result it is shown that grapes can produce different abilities to extract all phenolic compounds depending on their variety (Ortega-Regules *et al.,* 2006), and state of maturity (Ortega-Regules *et al.,* 2008). As a result, the differences witnessed in extractabilities has been related to the CWM within the cell wall as it acts as a barrier before releasing the phenolics present within the cell.

Water is fast becoming an expensive but necessary commodity throughout the agriculture industry. Therefore it is imperative to try to better understand the effect of water on the crop and the quality it produces before investing in possible irrigation.

The aim of this study is further understand berry development, and in particular of the development of the skin cell wall by assessing the effect of water availability on the cell wall composition. In addition to understanding the behaviour of two well known Portuguese grape varieties, Touriga Nacional and Trincadeira under different environmental conditions.

III. Materials & Methods

III.1 Experimental Vineyard

The study was conducted in a vineyard situated 70 kilometres east of Lisbon, Portugal, in the Peninsula de Setubal, at the Centro Experimental de Pegões (38°39'1"N 8°38'42"W), see figure 3.1.

Figure 3.1: Arial view of the experimental vineyard in Pegões, 70 km east of Lisbon

III.1.a Vines

Mixed clones of two cultivars, Touriga Nacional and Trincadeira, of *Vitis vinifera* L. were used. Both cultivars were grafted on 1103 Paulsen rootstock in 2002. Rows were spaced 2.5m apart, with 1m spacing between plants within a row. The vines were trellised on a vertical system with a pair of movable foliage wires to support upward shoot positioning. The vines were spur-pruned on a bilateral Royat Cordon (*circa* 12 buds a vine).

III.1.b Soil

The topsoil is generally dominated by podzolic soil and deeper, until 60cm in depth. Below this depth, the soil contained a predominantly sandy layer (60cm-1metre) with clay beyond 1 metre.

III.2 Treatments

Touriga Nacional and Trincadeira planted in one plot were treated to irrigation via drip emitters (4.0 l h^{-1}). There were two drip emitters located 30cm from the vine trunk on either side, supplying both sides of the root system. The irrigation supplied 100% of the crop's evapotranspiration (Etc). This treatment was labelled Fully Irrigated (FI) treatment. Touriga Nacional and Trincadeira were also planted in a

separate plot that was not irrigated (NI) and acted as the control. Three plants in each treatment and plot were preselected for analysis.

III.3 Climatic & Vineyard data

Meteorological data was sourced from the nearby meteorological station (http://www.meteo.pt/pt/oclima/acompanhamento/) for the period of 2010. The soil humidity was measured with a capacitance probe (Diviner 2000) in vertical tubes in the soil to a depth of 1m. The soil humidity was taken in each of the 4 treatment plots. Readings were taken in the early morning, 5 times over the growing season (27th July, 3rd August, 10th August, 24th August & 14th September).

The grapevine water status was assessed by the predawn leaf water potential. The water potential was determined by using a pressure chamber (Scholander *et al.,* 1965) at predawn. The assessment was carried between veraison and harvest (5th August) on the each of the preselected plants under the different treatments.

For berry sampling, 100 berries from each of the preselected plants were randomly selected at harvest on 20th September 2010. The harvest decision was based on when the grapes were deemed to have reached maturity in terms of sugar. All treatments were harvested on the same day.

III.4 Berry Composition Analysis

Once harvested and split into their groups (n=100, 3 samples per treatment), determined by which treatment and individual plant they originated from, the berries were immediately were taken to the laboratory to undergo berry analysis. The petioles were detached from each of the berries before being assessed for berry weight, berry volume, must pH, total solids (Brix^{o}) and total acidity (g/dm^{3}). The must pH was measure by using a calibrated pH meter, with the must at room temperature. The total acidity was measured via the titratable method with sodium hydroxide, and expressed in terms of tartaric acid.

III.4.a Physico-chemical Determination in Grapes

The colour intensity was expressed as a sum of absorbencies in a 1cm pathway at three different wavelenths (420nm, 520nm and 620nm), (Glories, 1984) by using a spectrometer.

III.4.b Total Anthocyanin

Total anthocyanin content was quantified through the decolourisation sodium bisuphate. The skins were suspended in buffered tartaric acid solution of pH 3.6. The solution contained a portion of 96% ethanol, with the volume being calculated by the total weight of fresh berries, divided by 8 to give the volume in millimetres ($Volume_{Ethanol}$(ml)= Total sample berry weight/8). The volume of tartaric pH 3.6 buffer was calculated by total berry volume subtracting the ethanol volume ($Volume_{pH3.6}$(ml)= Total sample berry volume – $Volume_{Ethanol}$). Thus replacing the original must volume with the buffer solution. Once left for 24 hours at 37^{o}C, the suspension was centrifuged (10,000 x rpm, 10 mins). 1ml of the supernatant was added to 1ml of 96% ethanol, and 20ml of 2% hydrochloric acid. The solution was mixed and split into two samples, with one sample having 4ml of distilled water being added and the other, 4ml of sodium bisulphate. The latter is the control due to the instant decolourisation. The differences in absorbencies were measured after 20 minutes at room temperature at 520nm. A standard curve was used to quantify the anthocyanin content with the absorbencies obtained (see *annex 1*).

III.4.c Total Phenolics

The absorbance at 280nm (Ribéreau-Gayon, 1970) was used to measure the total phenolic content. Following the skins being left in contact with the pH 3.2 buffer for 24 hours, 1ml of the solution was diluted with 100ml of distilled water before the absorbance were taken in a 1cm pathway at 280nm. The absorbencies were multiplied by the dilution factor (100 times) to give the total phenolic index.

III.5 Cell Wall Extraction

CWM was extracted from a adapted procedure (Vidal *et al.,* 2001 & Ortega-Regules *et al.,* 2006). The grape seeds were removed to leave just the skins. 10g of skins were lyophilised to form a powder form with liquid nitrogen. The ground skins were centrifuged twice (2,300 x g, 10 mins) with 40mM of sodium phosphate buffer pH 7 solution. The insoluble residues were treated with 30ml of ∝- amylase (15mg per 100ml) per sample and left agitating in a water bath at 37^{o}C overnight, following which the suspension was exposed to another centrifugation (2,300 x g, 10 mins). The pellet was washed twice with distilled water (2,300 x g, 10 mins) and left to dry at 37^{o}C overnight. The remaining insoluble residue was filtered (glass microfiber filter, ø47mm) with absolute acetone (3 x 30ml), methanol:chloroform (1:1, v/v; 3 x 30ml) and diethylether (2 x 25ml). The residue was left to dry at 37^{o}C overnight.

III.5.a Cellulose Quantification

The cellulose quantification within the CWM was determined by the Anthrone method (Dische, 1962). 5mg of the CWM (which equates to about 2-3mg of cellulose) was suspended in 3ml of acetic acid/nitric acid/distilled water solution (8:1:2, v/v). The suspension was heated for 30 minutes in a boiling water bath to hydrolyse the polysaccharides within the cell walls. The samples were cooled and centrifuged (3,500 x rpm, 15 mins). The pellet was washed twice under centrifugation (3,500 x rpm, 15 mins), once with water and once with acetone. The pellet was left in a 60^{o}C oven overnight to dry. The pellet was suspended in 0.2ml of 72% sulphuric acid, and left at room temperature for 3 hours. The solution was altered to 1M of sulphuric acid by adding 2.2ml of distilled water, and left for 3 hours at 110^{o}C. Filter paper was used as the standard.

1ml of 0.2% anthrone (in concentration sulphuric acid) was added to 0.5ml of the diluted (1:20 in distilled water, 1:100 for the paper) solution, before leaving in a boiling water bath (5mins). Once cooled, the absorbance was measured at 620nm against a standard curve produced by glucose at varying concentrations (see *annex 2*).

III.5.b Fractionation of Cell Walls & Dialysis

The CWM underwent fractionation with the addition of 10mg/ml (30ml) of 50mM of Trans-1,2-Cyclohexanediaminetetraacetic Acid (CDTA), pH 6.5, to 300mg of dried wall extract. The suspension was left for 8 hours with constant agitation at room temperature. The supernatant was retained after centrifugation (3,000 x rpm, 5 mins). The insoluble material was centrifuged once more after a further 8 hours of agitation with another 30ml of CDTA. The supernatant was mixed with the first fraction, before the pellet being washed with 30ml of distilled water (3,000 x rpm, 5 mins), with the supernatant being added to the previous collected to provide a CDTA fraction.

A separate fraction was produced with the addition of 22.5ml of 0.1M of potassium hydroxide (KOH), containing 20mM of sodium tetrahydridoborate ($NaBH_4$). The suspension was centrifuged (3,000 x rpm, 5 mins) after it was left to incubate for 18 hours with constant agitation. The supernatant was retained and the pellet was washed with distilled water under centrifugation (3,000 x rpm, 5 mins) with the supernatant being mixed with the previous – 0.1M KOH fraction. The addition of acetic acid decreased the pH to 5 to increase stability. A third fraction, 6M KOH, was collected by repeating the same steps above with 22.5ml of 6M KOH. However the agitation occurred in a water bath at 37^{o}C, and again acetic acid was added to reach pH 5. The residue in the pellet was left to dry in 50^{o}C.

Dialysis was carried out as a purification process to remove unwanted low molecular weight compounds from the fractions for the gas chromatography that was planned to be carried out. Each of the factions was placed into 40cm separate dialysis membrane strips (ø32mm). These were prepared by boiling the membranes in 10mM sodium hydroxycarbonate and 1mM ethylenediaminetetraacetic acid (EDTA) for 30 minutes. The membranes were cooled in 1mM EDTA at 4 degrees. Once tested for leaks, each fraction was poured into a membrane and sealed. These were left in constant slow moving distilled water, with a stirrer, overnight at 4^{o}C, changing the water only after the first hour. Volumes of each fraction were measured.

III.5.c Total Sugar Quantification

Total sugars were measured using a modified method with sulphuric phenol assay (Dubois *et al.,* 1956). Two hundred μl of phenol solution was added to a 0.2ml of sample solution (diluted to provide 10-100μg of sugars per sample) and agitated. 2

ml of concentrated sulphuric acid was added and agitated. The samples were left to incubate at room temperature for 30 minutes. Absorbance was measured at 490nm using glucose as the standard (see *annex 3*).

III.5.d Total Pectic & Neutral Sugar Quantifications

Total pectic quantification was determined by using a modified method (Blumenkrantz & Asboe-Hansen, 1973). Samples of the different fractions were diluted (1:1 for CDTA, 1:10 for 0.1M KOH, 1:2 for 6M KOH). 1.2ml of 12.5mM tetraborate in concentrated sulphuric acid was added to 0.2ml of the diluted sample solution in an ice bath. Once applied, the samples were left to incubate in a boiling water bath for 5 minutes and left to cool. Absorbance was measured at 520nm using galacturonic acid as a standard (see *annex 4* for standard curve). The neutral sugar content was calculated by being the difference in the total sugar absorbance and the uronic acid absorbance, and using the total sugar standard curve.

III.5.e Protein Quantification

A modification of the Bradford's method (Ramagli & Rodriguez, 1985) was used to quantify the proteins in the cell wall. Thirty mg of the CWM was suspended in 5 ml of 1M sodium hydroxide and left in the at water bath at 100^{o}C for 10 minutes. The suspension was diluted (1:100) with a diluted hydrochloric acid solution (1:800) and agitated for 5 minutes. Diluted Bradford's solution (1:2) was added to the sample solution in ratio 3:2. Absorbance was measured 590nm, with bovine serum albumin used as a standard to quantify total protein content (see *Annex 5*).

IV. Climatic Parameters

Under the Köppen Index, Portugal is classified to have a Temperate Climate (Group "C"), by having an average temperature of above 10°C in the warmest months (April-September in northern Hemisphere climates), with the remainder of the year having an average of between -3°C and 18°C. Under the same index, Portugal is classified to have a dry summer (Group "s") as during the summer, there is on average under 30mm of precipitation per month, and with the precipitation being less than one third of the wettest winter month of the same area. With the summer temperatures averaging above 10°C for four months, with the warmest month averaging over 22°C, categorised the Penisula de Setubal as hot (Group "a"). Concluding that the climate of the situated vineyard is categorised as a Mediterranean climate (Csa).

IV.1 2010 Climate

The year 2010 can be perceived as a typical year in terms of the climate experienced. The monthly minimum temperatures were lowest during the winter months, averaging below 10°C between November and April. During the majority of these months the vines undergo leaf fall and remain dormant. It is only when the medium temperatures increase above 10°C, in March, does the budburst occur and the growth cycle begin. The medium and maximum temperatures, mirror that of the minimum temperatures by increasing through the calendar year and peaking at average temperatures of 25°C and 33°C respectively. In accordance with the Köppen Index, the average temperatures from April through to September are over 10°C, with the remainder of the years average monthly temperature falling between -3°C and 18°C. With two months of July and August averaging over the required 22°C to coincide with the hot summer months parameters within the Köppen Index, with averages of 25°C and 24°C respectively *(Figure 3.1).*

The majority of the precipitation occurred within the cooler months with only 92mm of precipitation falling within the growing season of April to September (with the harvest date of 20th September), accounting for just 12.4% of the total annual rainfall. Therefore falling within the required precipitation parameter under Köppen Index, of less than 30mm per month in the summer. The months with most precipitation being December and February with 150mm and 125mm respectively with October, January and March accounting 100mm a month.

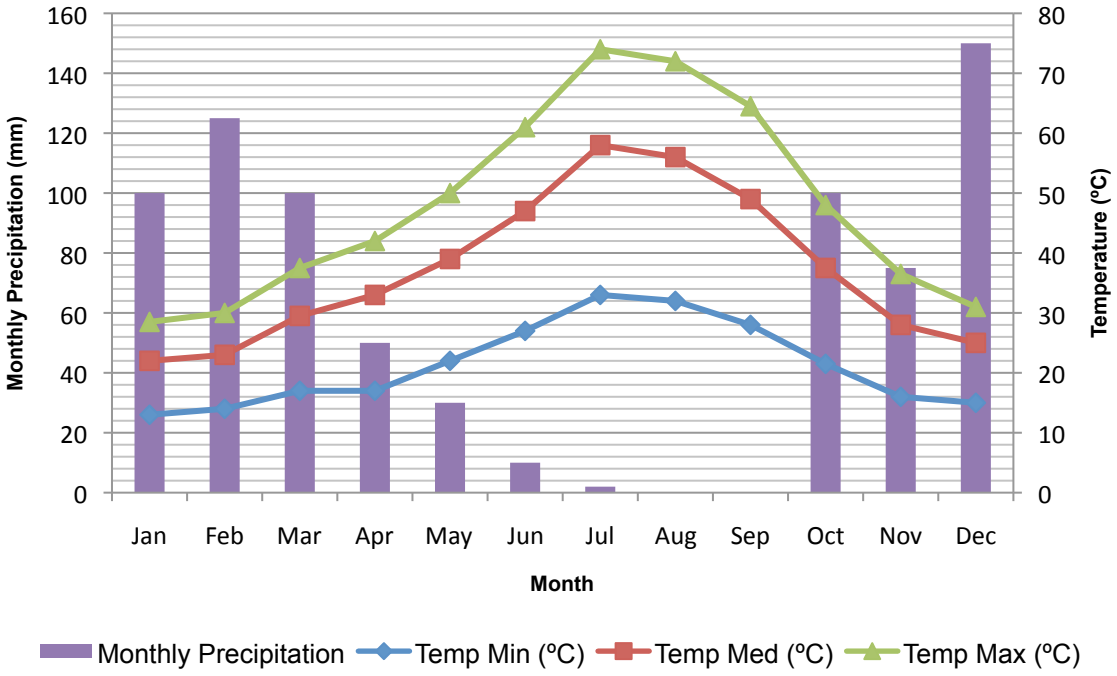

Figure 4.1: The monthly average maximum (Max), Medium (Med) and Minimum (Min) temperatures throughout 2010. The total monthly precipitation is also shown.

IV.2 Soil & Vine Water Status

Water levels in the soil, is shown as humidity present in the soil at different depths. Higher humidity suggests the water is more readily available to the plant. Despite different soils having different water holding capacities, the soil in this study is relatively homogeneous of loose sandy material until 90cm of depth, with low water holding capacity. Humidity in the soil around the fully irrigated plants had a much higher relative humidity than those of the non-irrigated.

Within the top soil (0-20cm of depth), the non irrigated plots showed low soil humidity values for both varieties, increasing from 0.399 and 0.601 at 0cm (surface) for

Trincadeira and Touriga Nacional to 7.45 and 5.58 at 30cm depth respectively. The lowest water availability is to be expected within the topsoil, as water is lost through soil evaporation from the high summer temperatures and wind, and water being absorbed from other plants with less extensive root system. At deeper depths, both Trincadeira and Touriga Nacional have similar and more stable soil humidity within the 4.20 and 5.63 respectively until 90cm (see *figure 4.2*).

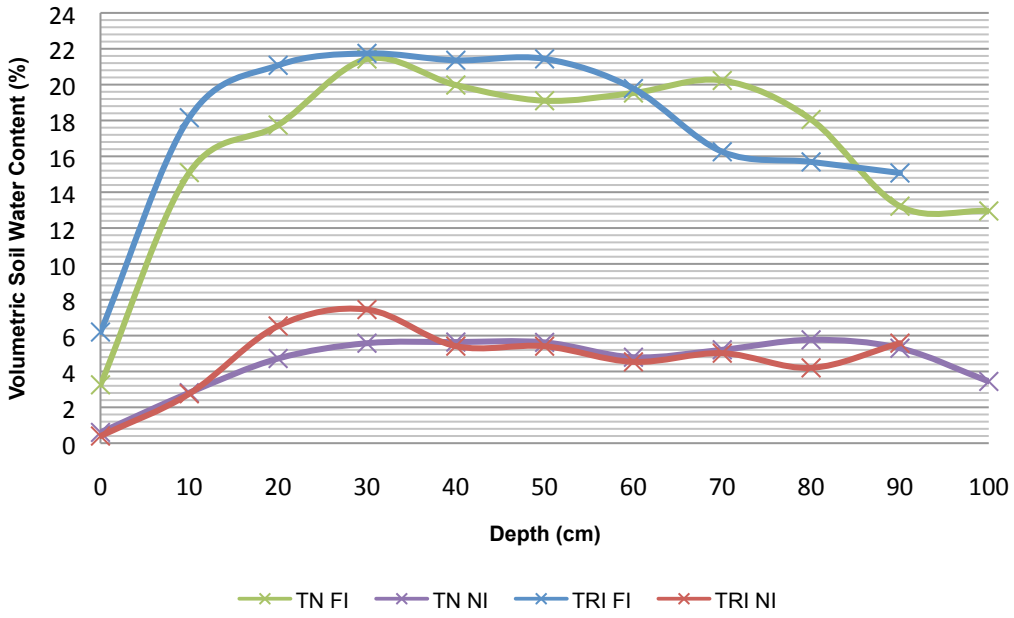

Figure 4.2: The average volumetric soil water content at different depths throughout growing season for the different treatments and varieties.

Both fully irrigated plots showed higher water content at all depths of the soil. At 0cm both varieties displayed the lowest water content within the treatment at 3.26% and 6.20% for Touriga Nacional and Trincadeira, before increasing and, like the non irrigated, peaking at 30 cm with 21.43% and 21.74% respectively. The increase in humidity within the topsoil is greater than that within the non irrigated the larger volume of water may be lost due to the soil water evaporation. The humidity remained stable until 60cm for Trincadeira and 70cm for Touriga Nacional, where at deeper depths, humidity decreased to 15.71% and 13.22% at 90cm. The higher water availability at all depths, within the fully irrigated treatment is to be expected due to the irrigation program.

During the maturation and before harvest, the predawn leaf water potential shows a clear difference in the water state of the plant between the two treatments of fully irrigated and non irrigated (*figure 4.3*). The vine water status of the vines under non-

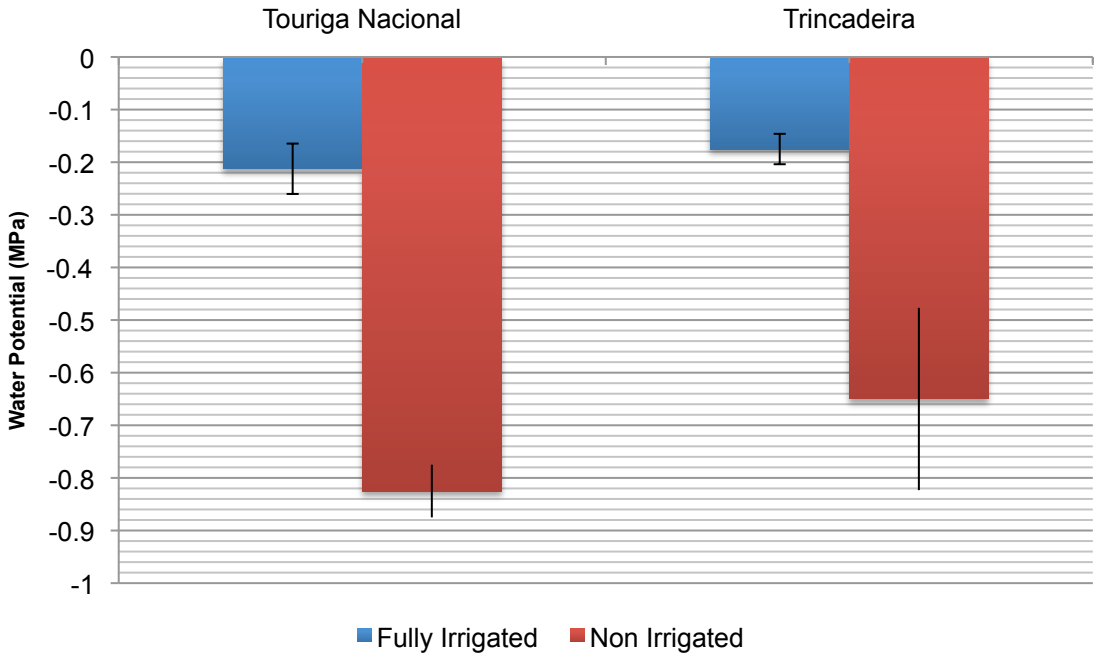

Figure 4.3: The predawn leaf water potential during maturation for Non Irrigated and Fully Irrigated and for Touriga Nacional and Trincadeira.

irrigated displayed a more negative water potential. The -0.825 MPa of non irrigated Touriga Nacional water potential is categorised to have a high water deficit (Carbonneau, 1998), whereas under the same categorisation, the non irrigated Trincadeira at -0.625 MPa is under severe to high water deficit. Both categorises show indications that the plant is suffering from a level of water stress.

For the fully irrigated, the Touriga Nacional displays mild to moderate water deficit and the Trincadeira showing no water stress with water potentials of -0.213 MPa and -0.175 MPa respectively. The threshold values are thought to be cultivar specific, which may give rise to the difference in categories, as for both fully irrigated and non irrigated the Touriga Nacional was only slightly over both threshold values of -0.8 MPa and -0.2 MPa.

V Results & Discussion

V.1 Berry Composition

The mean berry weights are shown to be significantly different between the two treatments within a variety. For the two varieties, the fully irrigated vines had heavier fruit with an increase of 0.4g and 1.06 of berry weight for Touriga Nacional and Trincadeira respectively over the non irrigated treatment of the two varieties (see *figure 5.1*). A similar trend is also seen with the same plots in 2011, with an increase from 1.70g for non irrigated to 3.40 for fully irrigated (an increase of 1.70g) with Touriga Nacional, and an increase from 1.61g to 2.83 for Trincadeira (an increase of 1.22g) (data not shown).

These values are in accordance with other studies as Touriga Nacional grown in non-irrigated plot has been found to be 1.30g and 1.70 under irrigated conditions (Nel, 2009). The trend of an increase with irrigation is also in accordance with other studies on other varieties, Cabernet Sauvignon (Koundouras *et al.,* 2009), Grenache Noir (Etchebarne et al., 2010), Tempranillo (Intrigiolo & Castel, 2010) and Syrah (Ojeda et al., 2001).

Berry volume also shows similar results with significant differences between treatments within a variety. With Touriga Nacional increasing from 0.69 ml per berry to 1.04ml for non irrigated and fully irrigated respectively. For Trincadeira, the non irrigated grapes had a volume of 0.77ml whereas fully irrigated had a volume of 1.57ml. Within the variety, fully irrigated vines produced grapes that were 49.9% and 103.6% greater than the non irrigated vines for Touriga Nacional and Trincadeira respectively.

Despite both varieties showing the same trend, the extent of the effect of the irrigation on both berry weight and berry volume appears to be different for both varieties. With Touriga Nacional increasing 31.4% and 49.9% in berry weight and berry volume respectively, whereas Trincadeira increased 78.7% and 103.6% for the same two parameters. Suggesting therefore that irrigation and water availability had a greater effect on these parameters within Trincadeira than in Touriga Nacional. The variation is to be expected as irrigation has been shown to have differing effects on

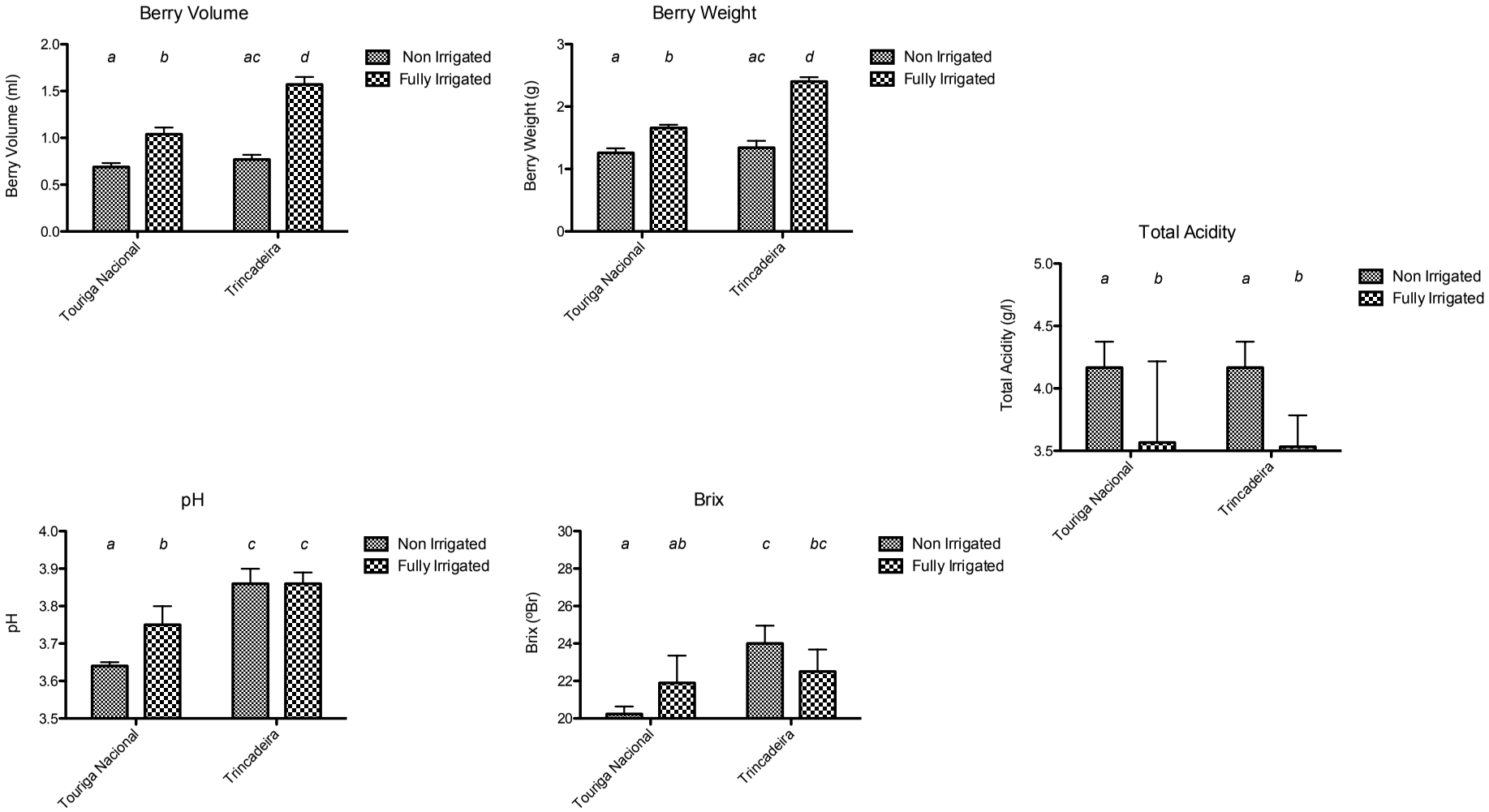

Figure 5.1: Figures displaying the grape composition under different parameters (Berry Volume, Berry Weight, pH, Brix and Total Acidity), for both Touriga Nacional and Trincadeira, for non-irrigated and fully-irrigated treatments. Different letters signify significant difference following an All Pairwise Multiple Comparison Procedure (Bonferroni t-test, P<0.001).

berry weight and volume in the previous studies. In addition, different years also affects the magnitude of influence irrigation has on berry size, as 2011 showed an increase of 5.4% and 10.7% in berry weight and berry volume for Touriga Nacional and 20.2% and 21.1% for Trincadeira (data not shown). In this case the difference is most likely due to the climatic conditions with 2011 being wetter, therefore nullifying the influence and need for irrigation, therefore having a lesser effect on berry size.

Between varieties show no difference with under non-irrigation. However under full irrigation, Trincadeira is shown to have significantly larger berries, by volume and weight. This suggests that irrigation has a greater effect with Trincadeira than Touriga Nacional on berry size, and this is most likely due to the vigour of the two plants, as Trincadeira was shown to have significantly more pruned wood than Touriga Nacional under both conditions (data not shown). However there was shown to be no difference in vigour between treatments within a variety.

There is a significant correlation (r value in excess of 0.98) between the berry volume and the berry weight (see *annex 6*) suggesting that the two variables are related. It has been widely reported that water availability affects berry growth (Ojeda *et al.,* 2002). The current findings are in accordance with previous studies in that water deficit leads to berries being produced with a lower weight. It is widely perceived that water deficit *preveraison* will cause an irreversible effect on the berry size, whereas water deficit only present *postveraison* is believed to be reversible later, if surplus water is made available later in the cycle.

Initial water deficit can lead to a decrease in cell division as the berry is connected to the plant through the xylem, therefore any water stress will lead to less water being transported to the berry, inducing a decrease in turgor within the mesocarp cells (Thomas *et al.,* 2006). In addition, water deficit *preverasion* decreases the cell division within this period that could lead to the irreversible effect on berry size. However, it should be noted, there is also evidence to show that cell division is unaffected (Ojeda *et al.,* 2001). Whereas the reversible *postverasion* reduction in size is thought to be a reduction in photosynthetic activity of the vine (Wang *et al.,* 2003), as during this period, the xylem is inactive and only the phloem supplies the berry. As the vine closes it's stomata when under moderate to high water stress (Chaves *et al.,* 2003), to mediate the water loss.

It is not possible to state the mechanism which led to the decrease in the berry size in this study as it is unsure when the non irrigated vines began to when the plants entered the state of water stress. Predawn leaf water potential was carried out in stage III of the growth cycle, between *veraison* and harvest, and the non irrigated vines were already showing signs of water stress. However water potentials do confirm that the non irrigated vines were under water stress, with both varieties having severe water deficit.

Brix a relative density scale used in winemaking where it indicates the percentage of sugar by weight in unfermented must. In this report, it was found that there was no significant difference between the treatments within the varieties. In addition, there appears to be a moderate increase with Touriga Nacional with water deficit, but not significant. It is thought that vigour may be a reason, with water deficit promoting the berry to become a more prominent carbon source with the relocation of carbohydrates by inhibiting lateral shoot growth (Coombe, 1989). However, studies have shown that the effect of sugar accumulation with water deficit is variety specific, as sugar content was seen to be significantly greater in Cabernet Sauvignon, but not with Merlot (Castellarin *et al,* 2007). However, as stated earlier, there was no difference in vigour through pruned wood weight between treatments. Therefore it should be considered that water deficit does not affect sugar accumulation with Touriga Nacional and Trincadeira.

There is a significant decrease in the total acidity of the berries under fully irrigated treatment, than with those in the non-irrigated. There is no difference between varieties, under both treatments, suggesting that irrigation has a similar effect on both varieties. However in contrast there is a significant difference between the varieties and the pH of the berries at harvest for Touriga Nacional for both the non-irrigated and the fully irrigated. In addition, Touriga Nacional also showed there to be a significant increase in the pH with irrigation. Water stress is shown to have conflicting effects it has on total acidity with some reports showing no overall effect (Matthews & Anderson, 1989; Esteban *et al.,* 1999) or an increase in total acidity (Santos *et al.,* 2007; Etchebarne *et al.,* 2010), or even a decrease with Tempranillo (Intrigiolo & Castel, 2010). However, studies in other regions within Spain have found pH to decrease pH (Esteban *et al.,* 2002) As it is thought that mild water stress and high temperatures can lead to malic acid degradation to berries with exposed fruit, thus lowering the total acidity (Kliewer, 1971). 2011 showed both a decrease in pH of the berries with irrigation and also an increase in total acidity (data not shown).

Reasons for results obtained in 2010 remain unexplained, but with 2011 results, one can only assume these results from one year not to be sufficient and samples from several years would prove more viable.

In appearance, the colour intensity significantly decreased with full-irrigation (see *figure 5.2*). With the reduction of the colour intensity being larger for Touriga Nacional than for Trincadeira. However there is also a significant difference between varieties within the same treatment, with Touriga Nacional showing significantly more colour than that of Trincadeira. Similar results have been achieved with Touriga Nacional and Trincadeira displaying colour intensities of 17.3 and 10.4 in non-irrigated plots (Abade & Guerra, 2008).

These results are also reflected with the decrease of anthocyanin content extracted at pH3.6 with irrigation (see *Annex 7)*. Both varieties showed that irrigation led to a decrease in anthocyanin content (see *figure 5.2*). In accordance with the colour intensity, there was a significant difference between varieties, with Touriga Nacional must having a higher concentration of anthocyanin content extracted at pH3.6 (see *Annex 8*). A decrease in wine and must colour has been documented as well as the decrease in anthocyanin content, with an increase of water availability (Mattews & Anderson, 1988, Santos *et al.,* 2005; Koundouras *et al.,* 2009; Intrigiolo & Castel, 2010, Sojo *et al.,* 2012). The correlation between colour intensity and anthocyanin content show that the anthocyanins are good indicators for the colour intensity of the wine or must (see *figure 5.3*).

Anthocyanins are synthesized through the flavanoid pathway within the berry skin of the red varieties. Water deficit is thought to induce the accumulation through the hydroxylation of certain anthocyanins (Castellarin *et al.,* 2007). This is done my converting the methoxylated anthocyanins (such as malvidin and peonidin) from their hydroxylated derivatives through the up regulation of the gene coding for the enzyme flavanoid 3',5'-hydroxylase (F3'5'H). It is also found that early water stress leads in an increased sugar accumulation that also increases the anthocyanin synthesis (Castellarin *et al.,* 2007).

Flavanols also play a key role in the colour of the wine or must through copigmentation. However the effect of water status and flavanol synthesis is fairly limited. Although water stress has been found to have a moderate increase in the

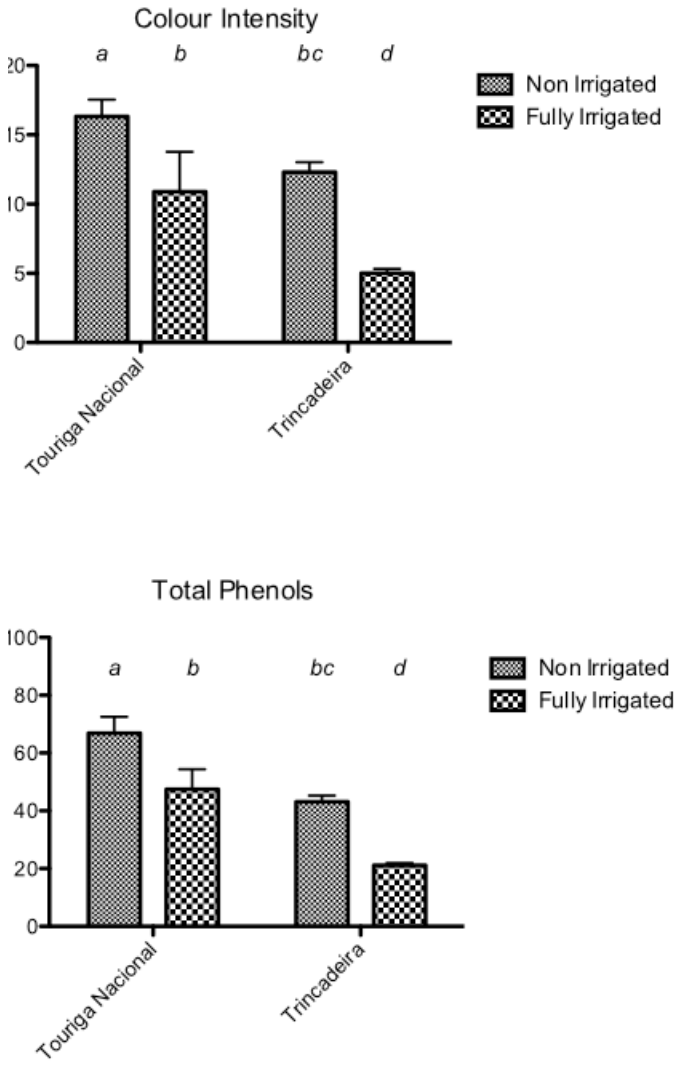

Figure 5.2 Figures displaying the grape composition under different parameters (Colour Intensity, Total Anthocyanin Content, andTotal Phenols), for both Touriga Nacional and Trincadeira, for non-irrigated and fully-irrigated treatments. Different letters signify significant difference following an All Pairwise Multiple Comparison Procedure (Bonferroni t-test, P<0.001).

flavanol synthesis (Grimplet *et al.,* 2007). And it has also been suggested that the flavanol and anthocyanin synthesis pathway may involve the same enzymes (Mattivi *et al.,* 2006). Even though this study did not focus on flavanol content, it should still be considered when assessing the colour intensity.

The anthocyanin content between varieties differ greatly as shown with Merlot, Mouvedre, Cabernet Sauvignon and Syrah (Ortega-Regules *et al.,* 2008), therefore a difference between Touriga Nacional and Trincadeira is to be expected. Additionally, differences in response to water availability have also been noted with Syrah only showing an increase in anthocyanin content when water deficit was induced *postveraison*. With the correlation with the colour intensity, it can be deduced that Touriga Nacional displays more colour and a reason maybe due to the higher content on anthocyanins over Trincadeira in Non irrigated and Fully irrigated. Previous studies have shown Touriga Nacional and Trincadeira to have slightly lower anthocyanin contents of 703mg/l and 429 mg/l (Abade & Guerra, 2008). Differences to levels in this study, may be due to the perceived maturity of the samples. In addition Touriga Nacional under fully irrigated is not significantly different to Trincadeira non irrigated. Therefore, Touriga Nacional is better suited under irrigation when compared to Trincadeira in terms of colour extraction.

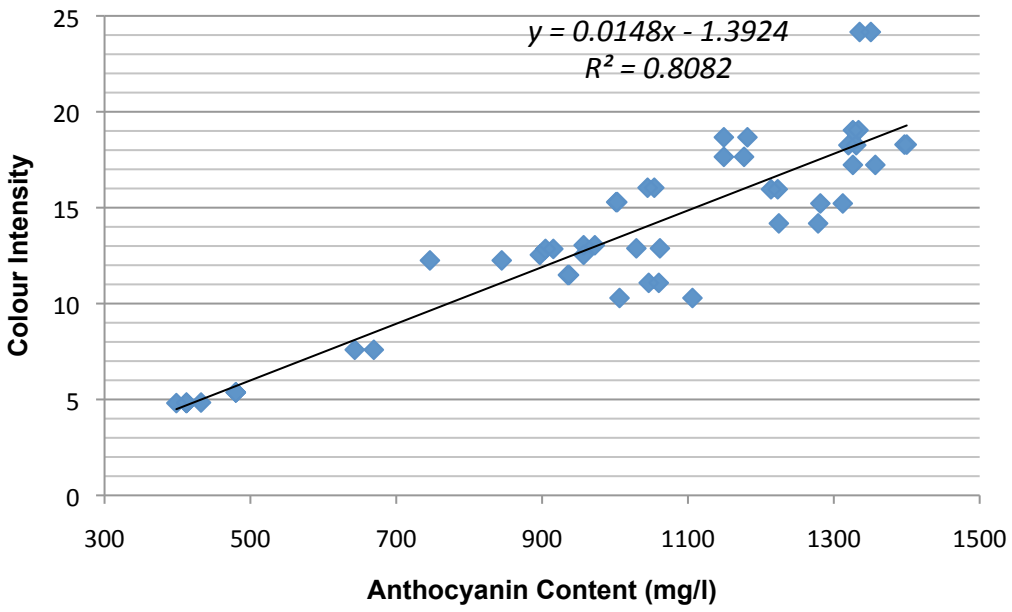

Figure 5.3: The correlation between total anthocyanin content in the skin of the grape berries with the colour intensity.

A decrease in total polyphenols was also seen with irrigation on both varieties, with Touriga Nacional decreasing from 66.9 to 47.4 with non irrigated and irrigated respectively and Trincadeira decreasing from 43.1 to 21.3 (see *figure 5.2*). Irrigation leads to a decrease of 29.1% and 51.0% of total phenols for Touriga Nacional and Trincadeira. This is in accordance with previous studies (Chaves *et al.,* 2007). This is to be expected due to the increase in anthocyanin content seen above under non-irrigation with a good correlation (see *annex 9*). It is unsure whether the increase is purely due to the fluctuations in anthocyanin content or other phenolics are the cause for the increase, including flavanols and proanthocyanidins. There are conflicting studies with flavanol levels with water availability, with some showing no difference with higher water availability (Sojo *et al.,* 2012) and some stating a decrease in skin proanthocynadins with irrigation (Downey *et al.,* 2006). Therefore further research is needed to better understand the behaviour of the grapes throughout maturation.

Touriga Nacional shows to have a significantly higher total phenol content than Trincadeira. Differences between the two varieties are in accordance with prior research where Touriga Nacional was shown to have an phenol content index of 71.06 and in the same study Trincadeira was lower at 36.4 (Abade & Guerra, 2008). This is to be expected as each variety is shown to have unique phenolic fingerprint and accumulation over the maturation period. This includes unique distribution of the phenolics within the berry tissues, and unique polymerisation degrees with Trincadeira, Touriga Nacional and Cabernet Sauvignon all having different accumulation of condensed tannins within the skins and the seeds, with Touriga Nacional having a wider range of polymerisation degree in both the skins and the seeds over Trincadeira (Cosme *et al.,* 2009).

V.2 Cell Wall Composition

Cellulose content for both Touriga Nacional and Trincadeira significantly increased with an increase in water availability (see *figure 5.4*). Touriga Nacional increasing from 129.0 μg/mg of CWM to 144.5 μg/mg, from non irrigated to fully irrigated respectively and Tricadeira increasing by a greater extent from 179.3 to 249.8 μg/mg of CWM.

This is in accordance with other studies that have also found that cellulose synthesis is highly sensitive to water availability, with water deficit showing a decrease in cellulose content (Iraki *et al.,* 1989; Sweet *et al.,* 1990).

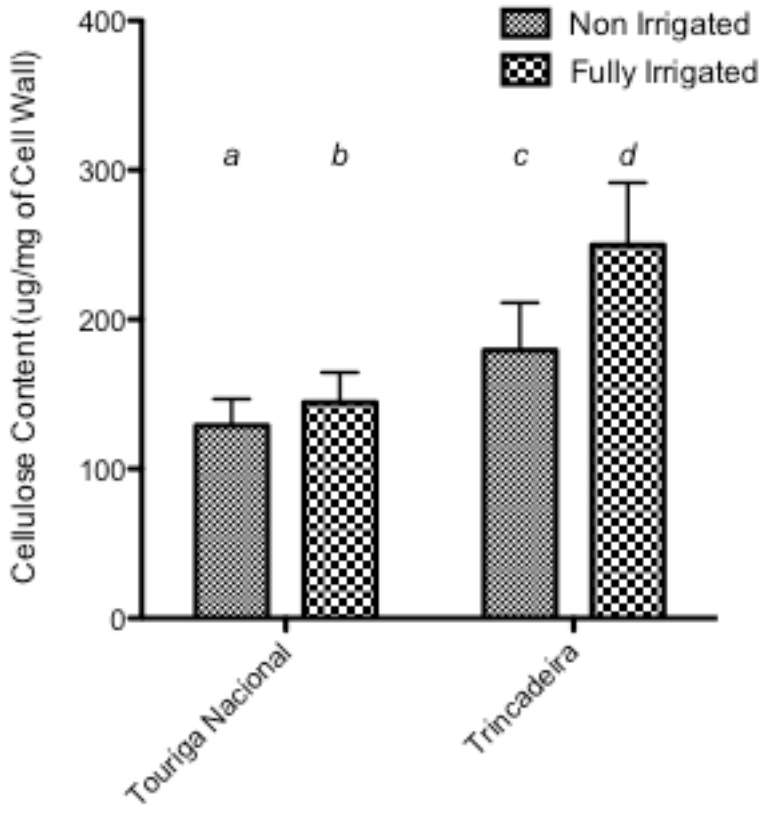

Figure 5.4: Total cell wall cellulose content (μg/mg of Cell Wall). Different letters signify significant difference following an All Pairwise Multiple Comparison Procedure (Bonferroni t-test. P<0.001).

Within both varieties for both non irrigated and fully irrigated, there was significant difference between the varieties. Touriga Nacional showed lower cellulose content for both treatments than Trincadeira. Differences between varieties have been found before with Monastrell showing to have significantly higher cellulosic glucose content than Cabernet Sauvignon, Syrah and Merlot (Ortega-Regules *et al.,* 2008).

In terms of total sugar content, there were no significant differences between the non-irrigated and the fully irrigated for Touriga Nacional within the CDTA fraction. There is a slight decrease from 10.1 to 8.52 μg/mg of CWM (see *figure 5.4*), in total polysaccharides extracted within the CDTA fraction but the difference was not deemed significant.

In contrast, within Trincadeira, the decrease from 10.6 to 6.46 μg/mg of CWM from non-irrigated to fully irrigated is significant. Between the varieties, there are no differences within the non irrigated treatment, with both showing very similar levels of total sugars extracted with 10.1 and 10.6 μg/mg of CWM for Touriga Nacional and Trincadeira respectively.

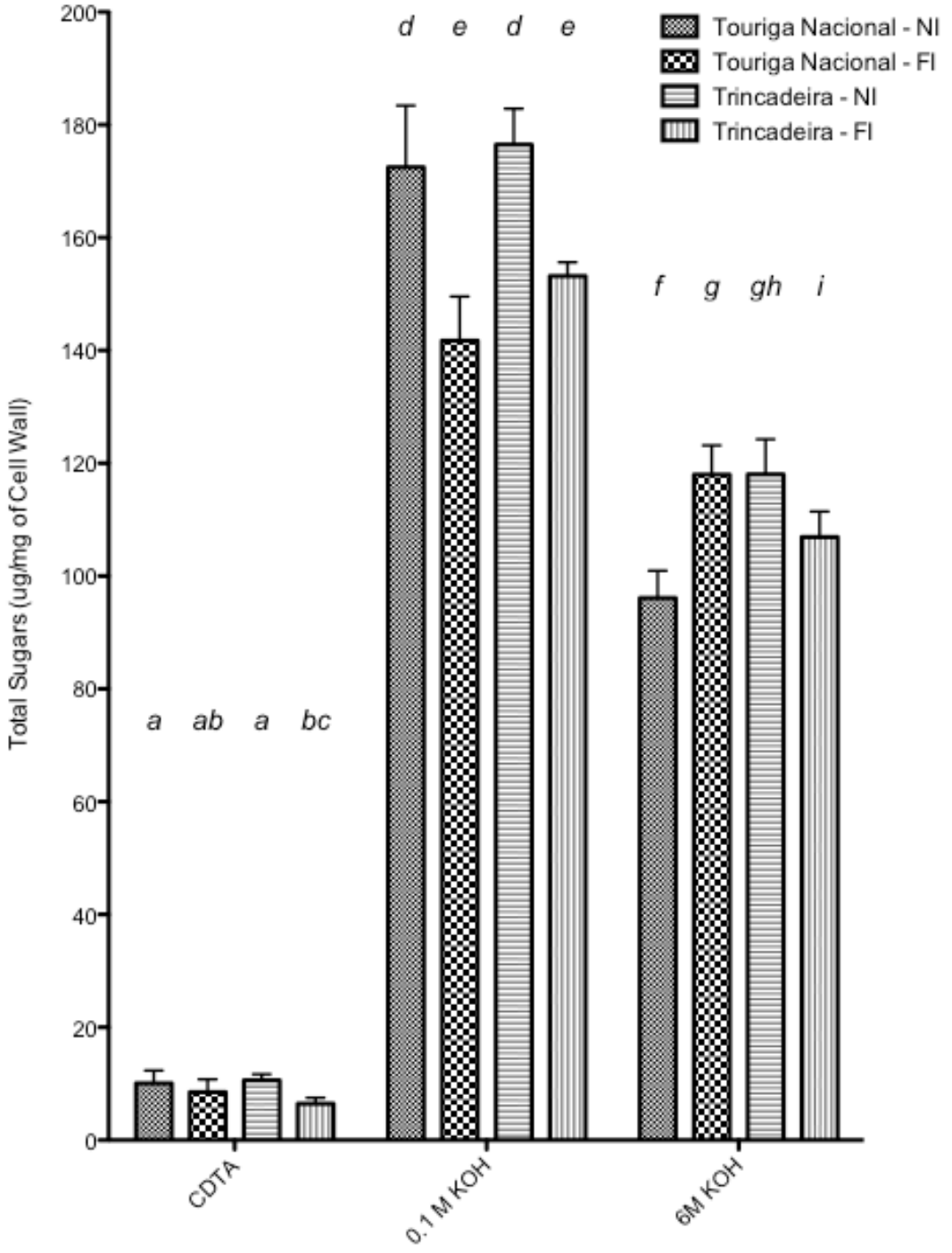

Figure 5.5: Different levels of total sugars extracted at different fractions of CDTA, 0.1M KOH and 6M KOH. Different letters signify significant difference following an All Pairwise Multiple Comparison Procedure performed at different fractions (Bonferroni t-test, P<0.001).

However, the decrease from the non-irrigated treatment to the fully-irrigated within Trincadeira was larger, suggesting that the effect of irrigation had a greater effect on the decrease of total sugar content extracted within the CDTA fraction (see *figure 5.5)*.

The sugars extracted at 0.1M of KOH were significantly different between non-irrigated and fully irrigated for both varieties. Total sugars extracted for Touriga Nacional decreased from 172.5 to 141.7 μg/mg of CWM whereas Trincadeira decreased by a smaller amount from 176.5 to 153.2 μg/mg of CWM. Within this fraction, there was no difference between varieties in the amount of total sugars extracted for both non irrigated and fully irrigated.

Within the 6M KOH fraction representing the sugars most tightly bound to the cell wall, water availability seemed to be responsible for the significant differences in the total sugars extracted in this fraction between the two varieties. Significantly higher content of total sugars were extracted in fully irrigated Touriga Nacional (118.0 μg/mg of CWM), when compared to the non-irrigated treatment (96.1 μg/mg of CWM). Whereas Trincadeira showed a different response to water availability, as the total sugar content extracted with the 6M KOH significantly decreased from 118.0 μg/mg of CWM in the non-irrigated treatment to 106.9 μg/mg of CWM within the fully irrigated treatment.

In most cases the content of total sugars extracted within a fraction and variety, decreased with the increase of water availability. Within a variety, the decrease was seen with the fully irrigated treatments in CDTA fraction for both Touriga Nacional and Trincadeira. The pattern was reflected within the 0.1M KOH fraction, with fully-irrigated treatments showing a significant decrease in total sugars extracted. Within the 6M KOH fraction, a decrease was also seen within Trincadeira with the fully-irrigated treatments. However, the opposite was observed with Touriga Nacional, with an increase in water availability significantly increasing the total sugars extracted at 6M KOH.

The decrease in total sugars extracted with the increase in water availability is contrary to previous studies that have found that water stress leads to a decrease in total cell wall sugars (Sweet *et al.,* 1990). However this does support the increase in total sugars extracted between non irrigated and fully irrigated treatments of Touriga

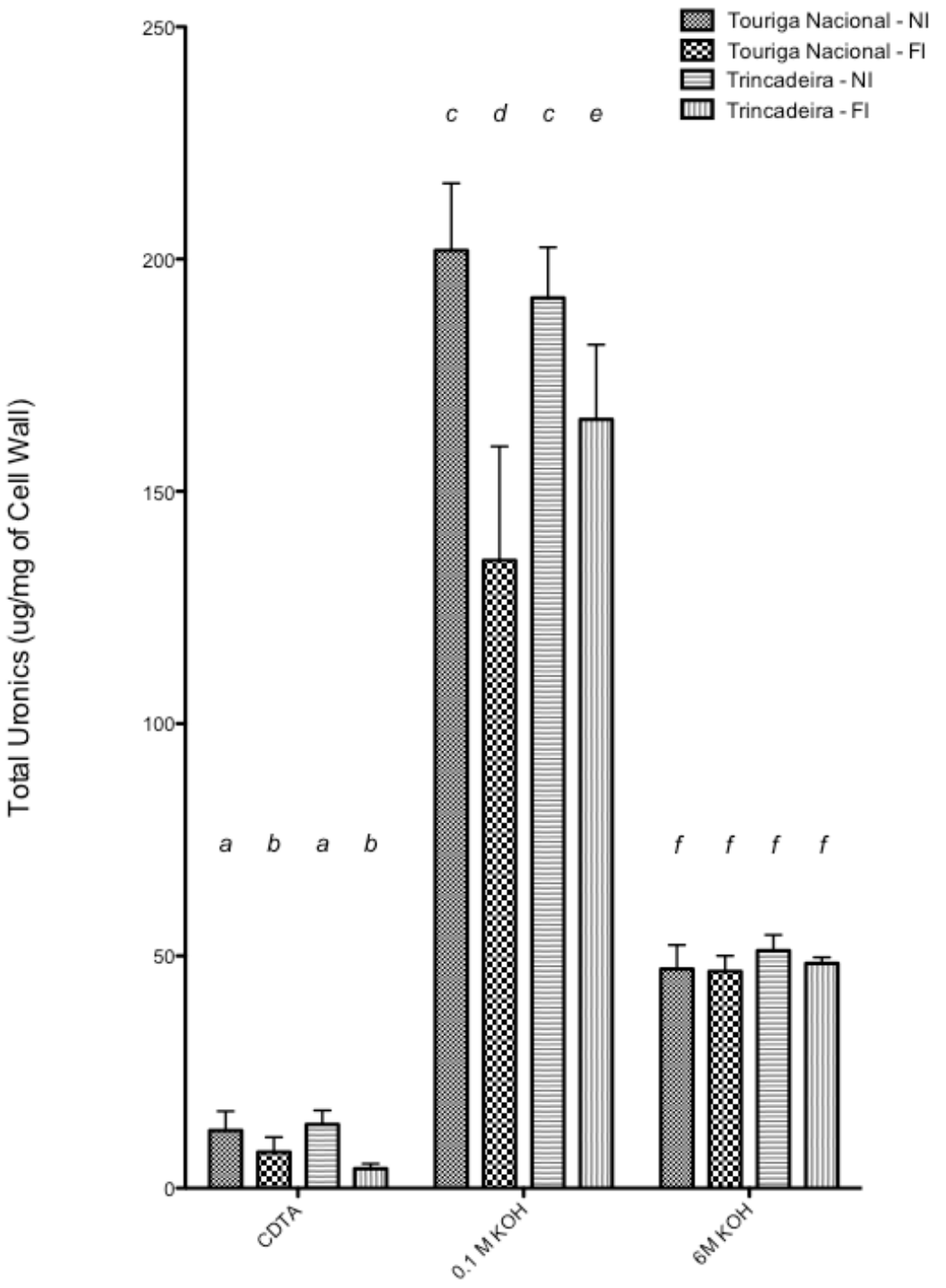

Figure 5.6: Different levels of uronic acids extracted at different fractions of CDTA, 0.1M KOH and 6M KOH, using galacturonic acid as a standard. Different letters signify significant difference following an All Pairwise Multiple Comparison Procedure performed at different fractions (Bonferroni t-test, P<0.001).

Nacional within the 6M KOH. As mentioned above, Sweet *et al.,* 1990 also found there to be a decrease in cellulose content as well as total sugars, within the leaf of the grapevine. The response observed within this study where the total sugars extracted is in contrast to the cellulose present within the different treatments. As the

decrease in cellulose levels with water stress supports the previous study, whereas the contrary increase in total sugars extracted under the same conditions is in stark contrast.

In terms of Touriga Nacional, the increase in the 6M fraction of total sugars extracted with irrigation, suggests that maybe that irrigation may provide more polysaccharides that are more tightly bound than non-irrigated. The KOH fractions predominantly extract the hemicelluloses (*eg* xyloglucans, arabinoxylans and (1-3,1-4)-β-D-glucans) from the insoluble residue by cleaving the hydrogen bonds and ester linkages present. The increase in concentrations means that different fractions are obtained as hemicelluloses have different bonding affinities.

Additionally, the composition of the pectins affect in which fraction they are extracted. Pectins are high in galacturonic acid content are generally extracted in the CDTA fraction. This is due to CDTA acting as a chelating agent, and is used to extract the calcium ions used in the cross-linking of the galacturonic acid chains, resulting in the galacturonic acids and the pectins to solubilise. Although, pectins with high neutral sugars are extracted in the KOH fractions.

However, there was a significant decrease in uronic acids extracted within the CDTA fractions when comparing the non-irrigated to fully-irrigated treatments for both Touriga Nacional and Trincadeira (see *figure 5.6*). The significant drop in uronic acid levels were also reflected in the 0.1M KOH fraction for both varieties, however at much higher levels. Suggesting that in this study, the majority of the uronic acids were extracted at 0.1M KOH, rather than CDTA fraction. In the 6M KOH, there were no differences between all treatments suggesting that any differences in pectin levels were extracted with the CDTA and 0.1M KOH fractions. For the majority, there were no differences between the varieties within the treatments, with the exception of Touriga Nacional and Trincadeira fully irrigated fractions at 0.1M KOH fraction, with a significant lower quantity of uronic acids extracted with Touriga Nacional than Trincadeira. Additionally, the decrease in uronic acid content between non-irrigated (201.9 μg/mg of CWM) and fully irrigated (135.2 μg/mg of CWM) with Touriga Nacional was larger than that witnessed with Trincadeira (191.6 μg/mg of CWM and 165.5 μg/mg of CWM for non-irrigated and fully-irrigated respectively).

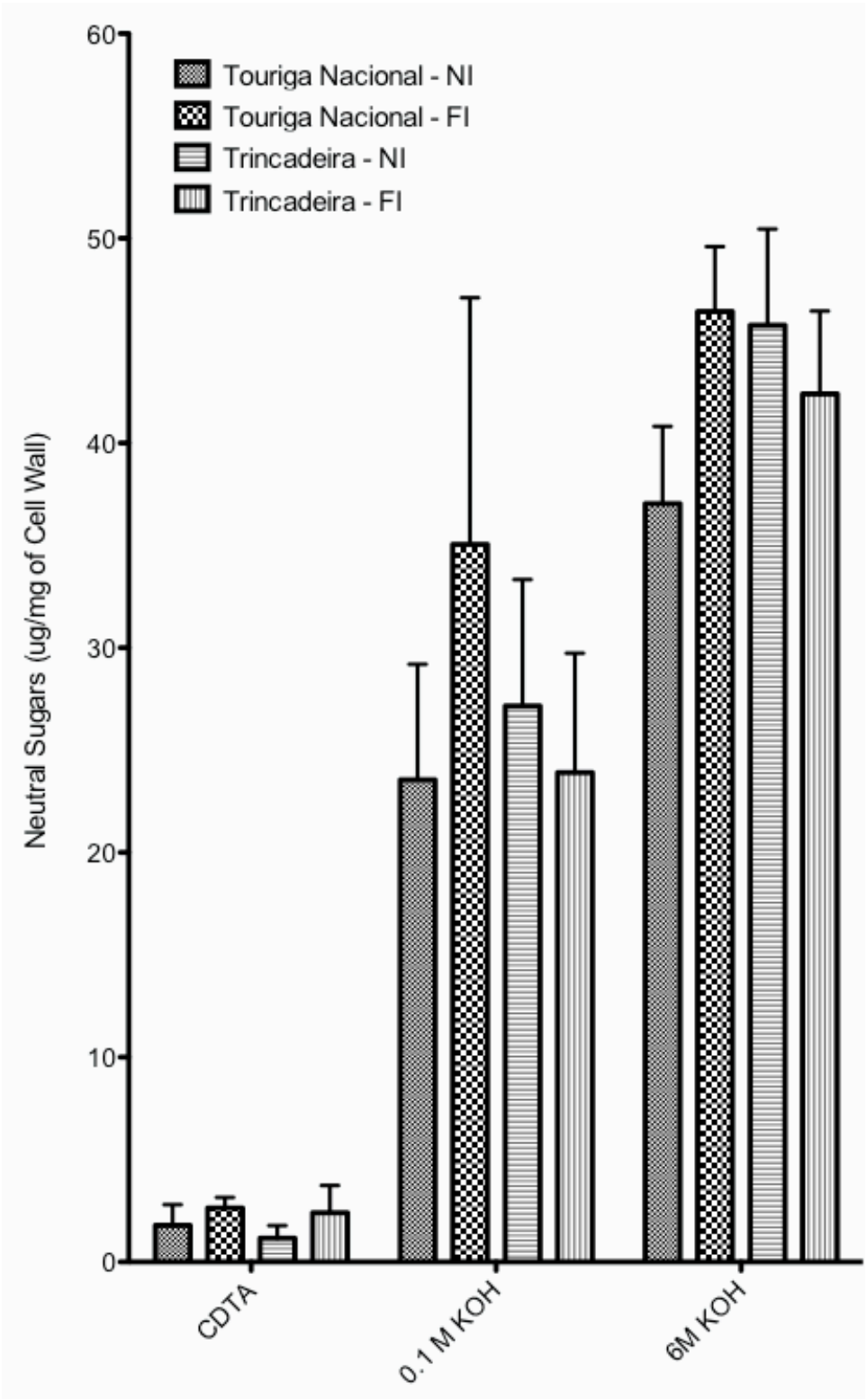

Figure 5.7: Different levels of neutral sugars extracted at different fractions of CDTA, 0.1M KOH and 6M KOH. Different letters signify significant difference following an All Pairwise Multiple Comparison Procedure performed at different fractions (Bonferroni t-test, P<0.001).

In terms of neutral sugars, there were no significant differences between almost all varieties and treatments within the CDTA fraction. However a difference was witnessed within both the 0.1M and 6M KOH fractions, with Touriga Nacional showing an increase in neutral sugars extracted from non-irrigated to fully-irrigated (23.5 μg/mg of CWM to 35.5 μg/mg of CWM and 37.0 μg/mg of CWM to 46.4 μg/mg of CWM respectively for 0.1M KOH and 6M KOH). Whereas the opposite response was witnessed with Trincadeira with a decrease in neutral sugars in both fractions from non-irrigated to fully irrigated (27.2 μg/mg of CWM to 23.9 μg/mg of CWM and 45.8 μg/mg of CWM 42.4 μg/mg of CWM for 0.1M KOH and 6M KOH respectively), however the decrease was not significant. It can be deduced that in terms of Touriga Nacional, that the pectins present in fully irrigated may have a lower uronic acid content which is mirrored with a higher neutral sugar content. Whereas higher water availability with Trincadeira, shows to decrease both the uronic acid (see *figure 5.6*) and the neutral sugar contents (see *figure* 5.7).

It is also widely acknowledged that fruit softening is associated with changes in the cell wall composition. With the grape skin, changes in pectic polysaccharides through the losses of AG-II and PG are seen throughout ripening (Nunan *et al.,*1998) and a decrease in neutral sugars are usually seen (Barnavon *et al.,* 2000) as well as a decrease in hemicellulose and cellulose levels (Yakushiji *et al.,* 2001). Whilst some have found that cellulose levels remain unchanged (Nunan *et al.,* 1998; Ortega-Regules *et al.,* 2008). Although it was not possible to determine the quantity of pectins in the present study and how they were bound, it is reported that methylation of the pectins decreases throughout maturation (Ortega-Regules *et al.,* 2008; Vicens *et al.,* 2009) and a decrease in uronic acids have been witnessed in other fruit such as different strawberry cultivars (Rosli & Civello, 2004).

Therefore it must be noted that any changes in the uronic acids may be attributed to the degree of maturity of the berry, with fully irrigated possibly achieving a higher degree of maturity at harvest than non irrigated giving rise to lower uronic acid content. In addition, maturity is also associated with a decrease with neutral sugars, especially galactose (Ortega-Regules *et al.,* 2008; Vicens *et al.,* 2009). This also supports that theory that the full-irrigated berries were displaying signs for higher degree of maturity with the decrease of neutral sugars with an increase of water availability with Trincadeira. The increase in water availability may have provided the

vine with sufficient water to achieve an improved carbon balance so the fruit can achieve maturity more readily, over non-irrigated vines. Individual sugars were not identified in this study, so it is not possible to determine the state of maturity in terms with the decrease of galactose, this is recommended with the use of Gas Chromatography (GC) or HPLC. A possible method to overcome this is to choose a harvest index (*eg* Brix/Total acidity) as a measure, and when a harvest when the index is displays the same result.

However, it must be noted that changes in the cell wall composition over the ripening period is very variety dependent (Ortega-Regules *et al.,* 2008) giving rise to differences between Touriga Nacional and Trincadeira. In terms of cell wall protein content (see *figure 5.8)*, there was no difference between the different treatments and varieties. There were minimal differences witnessed between the treatment, but not significant. Differences in cell wall proteins have been witnessed between varieties (Ortega-Regules *et al.,* 2006) with Cabernet Sauvignon and Merlot having differing levels, whereas Cabernet Sauvignon had similar levels to Syrah. Therefore it may be deduced that Trincadeira and Touriga Nacional have similar cell wall protein levels.

Structural proteins play an important role in the ripening of fruit, including grapes, as they are vital in the alteration of the composition of the cell wall, changing is chemical and physical properties. The major family of proteins include, extensins, glycine-rich proteins (GRPs), proline-rich proteins (PRPs) and arabinogalactan proteins (AGPs) (Showalter, 1993). Extensins are the most influential in terms of cell wall functions as they are Hydroxyproline Rich Proteins (HRP) and have the ability to covalently bond to polysaccharides. Throughout ripening, there is a large increase in amino acid content within the cell wall *postveraison* (Nunan *et al.,* 1998; Vicens *et al*., 2009). This correlates with the increased amount of PRP and hydroxproline-rich glycoproteins after *veraison* (Davies *et al.,* 2000) suggesting that the extensins are synthesised and incorporated into the cell wall *postveraison*. As a result as there is a partial loss of structural polysaccharides within the cell wall of the skin, it is compensated with the insertion of structural proteins to ensure that the berry maintains its integrity and protective tissue.

In addition, throughout maturity, the cell wall softens through the increase in cell wall enzymatic activity. A dramatic increase in wall-bound peroxidases have been found (Calderon *et al*., 1993) within the outer four cell layers, including the epidermis and

subepidermis (Huang *et al.,* 2005). This provides an irreversible formation of phenolic cross-linking between structural proteins and polysaccharides, which provides the cell wall with strength.

The loss of galactose, and in particular residues from the AGI, is associated with cell wall softening during ripening, is most likely due to enzymes, as β-galactosidase activity has been shown to increase and probably has a important role in the hydrolysis of cell wall galactan during berry development and role in berry skin softening (Nunan *et al.,* 2001). In addition, α- galactosidase activity also increases *postveraison*, and is thought to be vital in the development of the cell wall during maturation, although it is unsure what the substrate of this enzyme truly is.

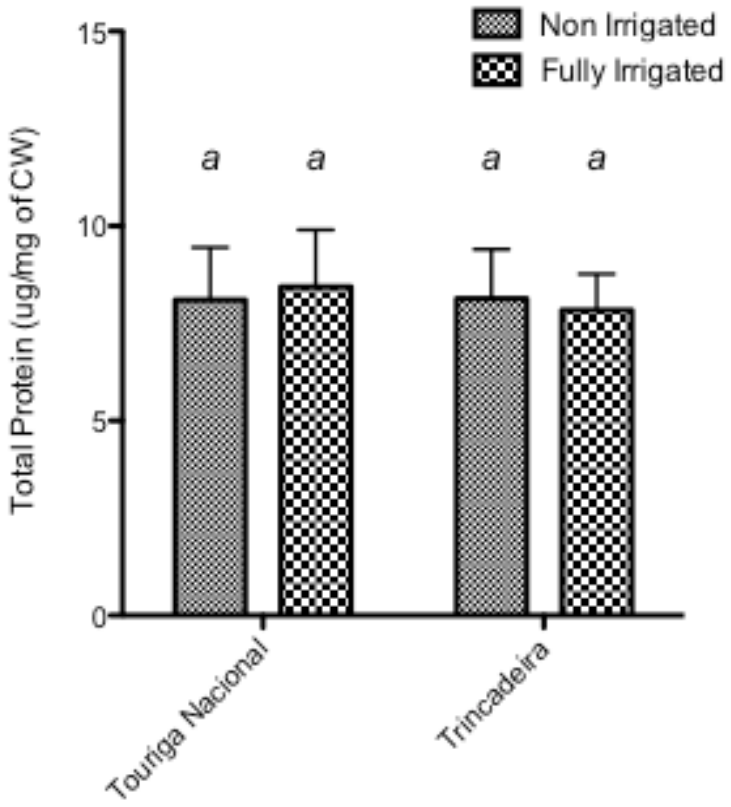

Figure 5.8: Cell wall protein content for Touriga Nacional and Trincadeira for non-irrigated and fully-irrigated treatments. Different letters signify significant difference following an All Pairwise Multiple Comparison Procedure (Bonferroni t-test, P<0.001).

There has been little work on cell wall proteins and their association with water availability. With tobacco leaves, osmotic stress was shown to not to have a great effect on the overall protein content present in the cell wall, however, in terms of the composition, there was generally no change in the proline and hydroxyproline-rich

amino acids, whereas unaffected conditions saw a marked increase in the amino acids, like witnessed with the maturation of grapes (Iraki *et al.,* 1989). In this study, the composition of the protein content was not assessed, therefore it is impossible to state whether the proteins present are rich in proline and hydroxyproline glycoprotein content. In addition, cell wall proteins were higher in Monastrell grapes from a drier region than those grown in a region with larger rainfall, although one cannot deduce that water availability was a determining factor (Ortega-Regules *et al.,* 2006).

The skins were exposed to very harsh conditions throughout the experiment, having been incubated in solutions containing pH3.6 tartaric buffer solution for 24 hours and pH1.0 hydrochloric acid for 4 hours respectively. As a result, the composition of the skins was most likely modified throughout this period, especially in terms of the polysaccharide content and the protein content. Proteins exposed to extreme conditions are prone to denature, including strong acid conditions that are most likely to break linkages within the tertiary structure and maybe causing them to solubilise. This could give rise to there being no difference between treatments in terms of protein content. Similar concerns may be determined with the polysaccharides, however these seem to be less affected as they bound together more tightly producing a more stable structure under such conditions.

VI. Final Considerations

Grapes are one of the most cultivated fruit in the World by area and volume. Much of this is due to versatility of the fruit to grow and show different characters when grown in different environments. Now, in an age where information is more readily available, consumers are becoming more inquisitive and knowledgeable about the traceability of the wine and grapes. As a result there is pressure to understand the science behind an ancient trade and form of agriculture to an already a highly competitive business. Understanding the science behind winemaking could optimise both grape and wine, whilst also possibly finally reducing production costs.

Success of Portuguese wines on a global scale has led to an increase in discovering the characteristics of some of the many native varieties, including Touriga Nacional and Trincadeira. Therefore it is important to understand how these varieties perform under different environmental conditions to optimise the wine quality from the potential of the grapes.

Additionally, water is one of the World's most sought after resource in terms of agriculture. Especially as average temperatures in some winemaking regions of the World are on the increase along with drought. A lot of focus has been on the ability of *Vitis vinifera* to survive and behave under different levels of water stress and the quality of grapes produced. As it has been found that combined effects of drought, hot temperatures and high evaporative demand of the plant during the growing season is known to limit the yield and grape and wine quality (Chaves *et al.,* 2007). Irrigation has been seen as a possible solution to these problems, however it has been shown that irrigation may be detrimental to wine quality (Bravdo *et al.,* 1985).

It has been well documented that irrigation affects berry composition, from berry size to total phenolics present. Increased water availability is shown to increase berry size and weight, as well as decreasing the total phenolics within the berry. In terms of the varieties in question, increased water availability had a greater effect on Trincadeira with the phenolic compounds decreasing significantly and the berry size increasing by a greater amount. As a result, this led to a decrease in anthocyanin content and colour intensity. The smaller berry size due to water deficit leads to a less fresh berry mass and a higher skin to pulp ratio. Considering large portions of the phenolic compounds are localised in the skin and seeds, this leads to potentially extracting

more of the phenolic compounds per volume of must. The phenolic compounds are perceived to affect the quality of red wine produced. Anthocyanins, for most varieties are predominantly found in the vacuoles of the berry skins, provide colour to the wine. Whereas proanthocynanidins, extracted from both the seeds and skins, provide body to the wine, and flavanols further stabilise the colour with co-pigmentation with the anthocyanins providing a potential for the wine to age.

The high anthocyanin content and ability to maintain a relatively small berry size under irrigation could be a possible reason for the recognition Touriga Nacional is receiving as a variety to potentially produce high quality red wine. Additionally, Touriga Nacional has a high content of carotenoids, a precursor of C13-norisoprenoid compounds that provide varietal aromas to the wine, which are also located within the skin of the berry. It also must be said that under increased water availability, with irrigation or different water retention abilities of different soils, there was a decrease in carotenoid content within the skins and therefore potential aromas (Oliveira *et al.,* 2003).

Understanding the demands of the vine is vital in deciding whether irrigation is recommended or not. In this case, the vigour of the vines remained relatively unchanged between non-irrigated and fully-irrigated for both Touriga Nacional and Trincadeira through pruned wood weights (data not shown). With Touriga Nacional showing 404g and 413g per metre for fully irrigated and non-irrigated, and Trincadeira showing 692g and 683g respectively. This suggests that Trincadeira was more vigorous in this situation, with Touriga Nacional possibly providing a better microclimate for the berries to mature in terms of phenolics, as exposure is known to improve grape quality (Smart *et al.,* 1990), as well as varietal differences.

Tissue softening is one of the major characteristics of fruit ripening. This is largely due to the dissolution of the cell wall through reorganisation and evolution of the polysacharides within the complex structure. Understanding the mechanisms behind the changes in the cell wall structure is vital in terms of red winemaking as it provides a barrier for the extraction of certain phenolic compounds that are beneficial to the wine, namely, anthocyanins, flavanols and proanthocyanidins. Red varieties with high in phenolic compounds do not necessarily mean that the must will contain also high levels following maceration, suggesting a something affects the transfer rate.

Throughout the ripening period, changes in the cell wall composition has been witness in terms of hemicellulose and pectin content as well as protein levels. In this study different environmental conditions in terms of water availability are shown to have an effect on the cell wall composition. Cellulosic glucose content is shown to decrease in vines under water stress, and the magnitude of impact is variety specific. A decrease in pectin content has been witness with ripening, by a minimal amount for strawberries (Rosli & Cevello, 2004) whereas in grapes it has been found to not change significantly (Ortega-Regules *et al.*, 2008). This study suggests that water availability did not change significantly the pectic content present in the skins for both varieties. There was a decrease in uronics extracted within the CDTA fraction, suggesting that the cell methylation degree with calcium ions of the pectins decreased with an increase in water availability. The same is also seen with ripening of the berry (Ortega-Regules *et al.,* 2008) and associated with the softening of the wall. This could be a possible explanation for the increase in extractability noticed with berries from irrigated vines for both varieties.

Both varieties displayed different responses in neutral sugar content to water availability. In terms of the ripening period, neutral sugars are seen to decrease, associated with the decrease in galactose (Ortega-Regules *et al.,* 2008) as well as the solubilsation of AGP-I. This may provide the decrease in the neutral sugars seen in Trincadeira with the presence of irrigation. However the opposite is seen in Touriga Nacional. This maybe due to a possible accumulation of free cellulosic glucose that can also occur during ripening. However it was not possible to confirm this without further analysis with HPLC or GC.

Although there were no differences seen in protein levels, one can assume that there were fluctuations present, but due to the methodology of the experiment, the effect was nullified. As proteins, such as extensins and hydroxyproline-rich proteins, are seen to be incorporated into the cell wall to compensate the loosening of the polysaccharides to ensure the cell wall retains its integrity (Nunan *et al.,* 1998).

However, much of the changes in the cell wall polysaccharide structure and composition is due to enzymatic activity. β-galactosidase is associated with the solubilisation of galactose/galactan and AG-I content, as the enzyme is present in all stages of berry development, with it's activity increasing significantly prior to cell wall softening. In terms of pectins, pectin methylesterase has also been found, along with polygalacturonase and pectate lyase (Nunan *et al.,* 2001) being associated with the

increase of solubility of PG. It has also been found that, with the loss of cell wall extensibility, there is an increase in peroxidase activity, especially in the outer four layers of the skin (Calderon *et al.,* 1993; Huang *et al.,* 2005). This enzyme is responsible for the formation of phenolic cross linking between structural proteins bridges, such as extensin, with isodityrosine and pectin or hemicellulose chains through diferulic bridges. The determination of structural proteins and enzymes was out of the scope of this study; however it is recommended to determine the effect of water stress on further protein identification and the expression of the respective coding genes.

Enzymes are commonly used in vinification to facilitate the extraction of some phenolic compounds throughout macerations. Pectinases are generally the most commonly used throughout the maceration phase of winemaking as it has been shown to increase tannin colour extraction from the grapes (Bautista *et al.,* 2005).

In addition there is also an increase in pectins within the must, with the use of pectinases. This will have an effect on both the winemaking treatments and the sensorial ananlysis. Pectic substances have been found to have high retention rate during filtration. They also have a positive note in that AGPs in white wine have a protective effect of protein casse. Additionally, both RG-I and RG-II act as a tartaric crystallisation inhibitor in wine, as well as polyphenols in red wine. However, sensorily, the pectins can combine with tannins increasing the perceived astringency.

Although no direct conclusions can be made from this study as further analysis in identifications of sugars, phenolics and proteins is needed, however, it has been shown that water status of the grapevine has consequences on the structure and composition of both the grape and the cell wall of the grape skin. Changes seem to be universal between the two varieties of Trincadeira and Touriga Nacional, however some differences were noticed suggesting some varietal differences. It is important to understand the effects of water status on the berries, as it can potentially alter the quality of wine produced and alter the techniques used in the winery.

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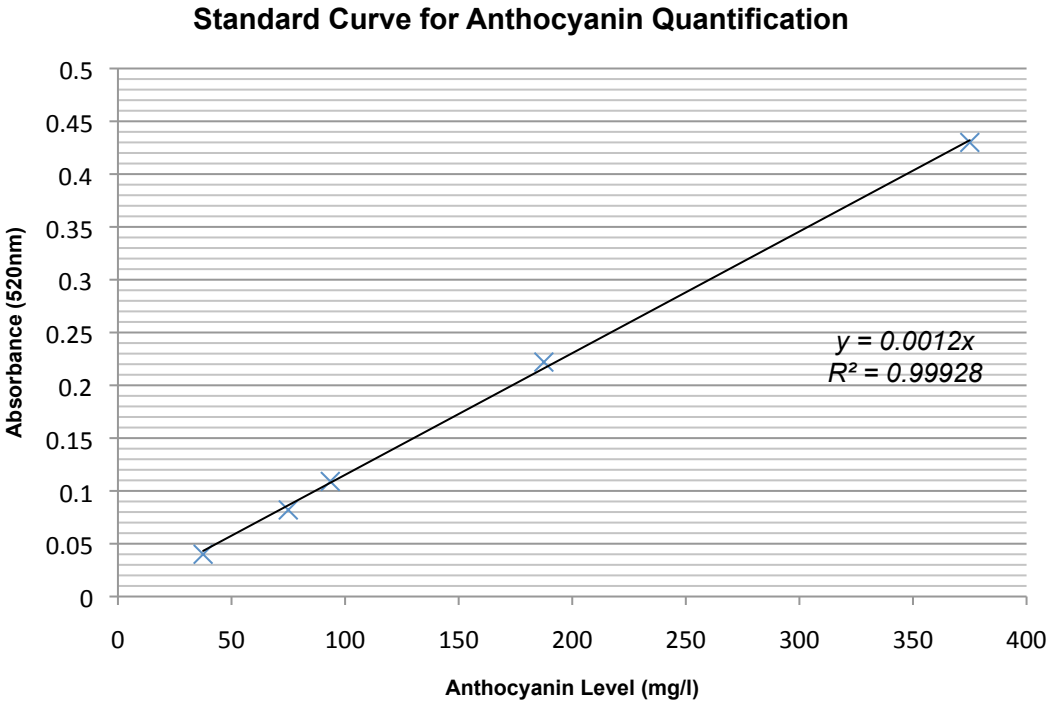
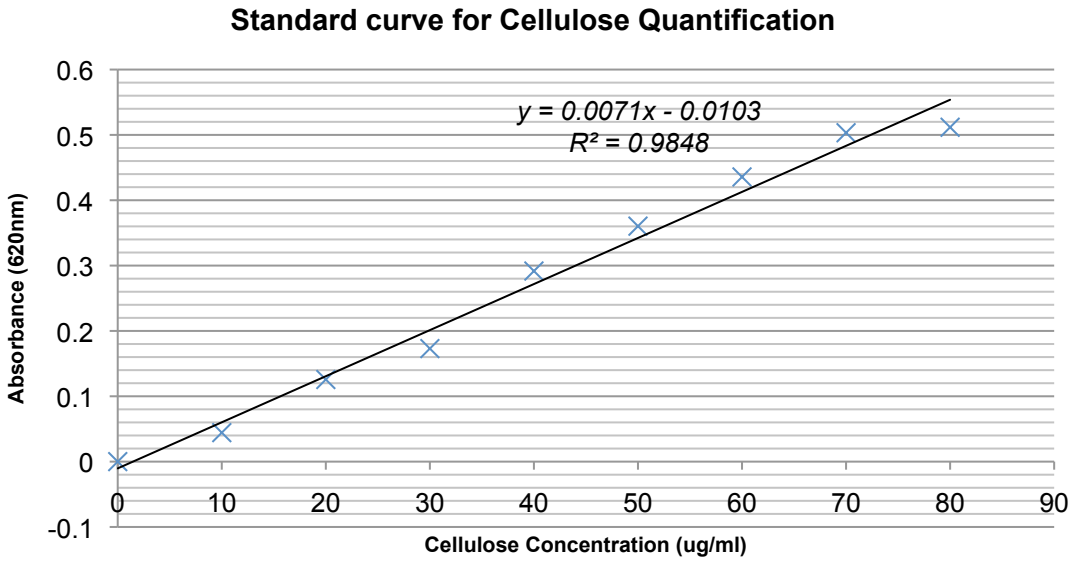
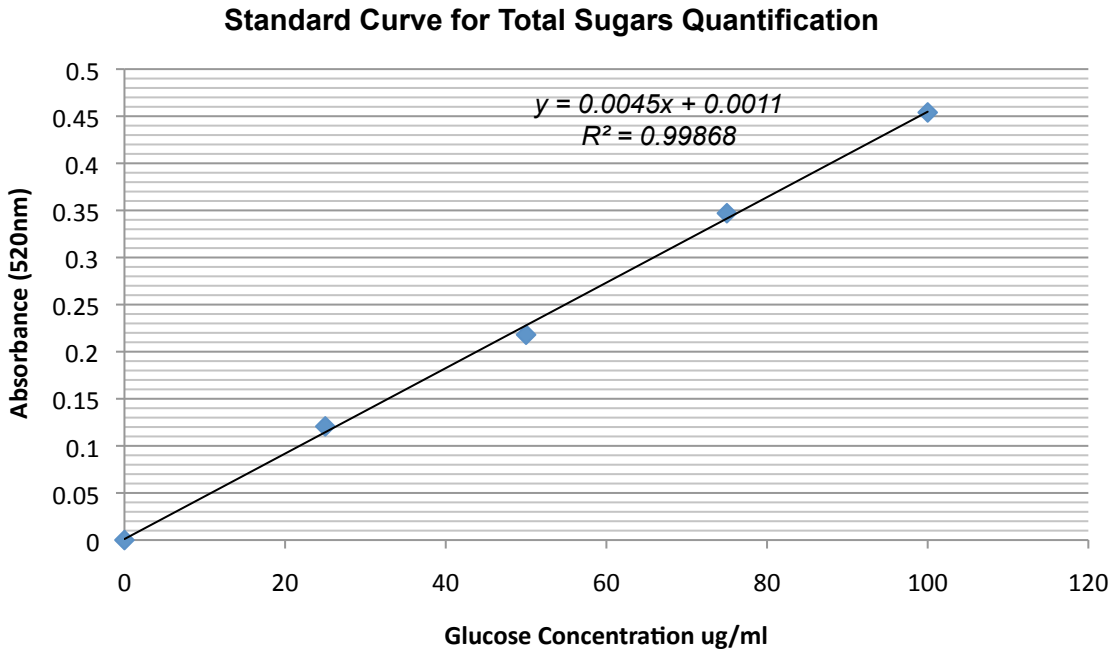
VIII. Annex



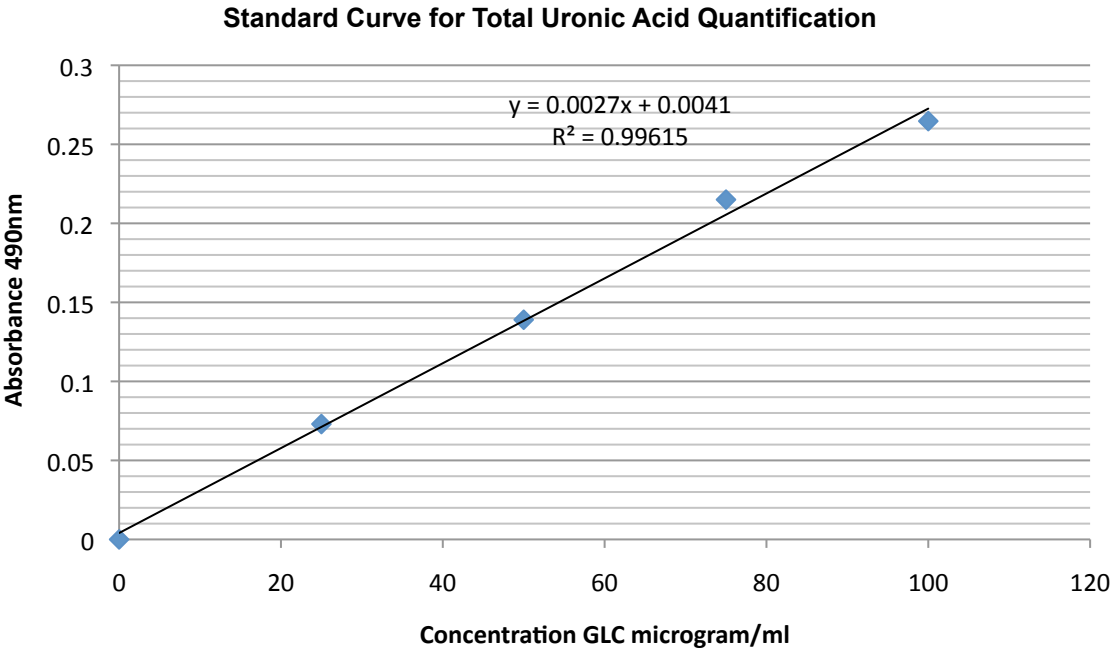
Annex 1: The standard curve used to determine the anthocyanin content in the skins.



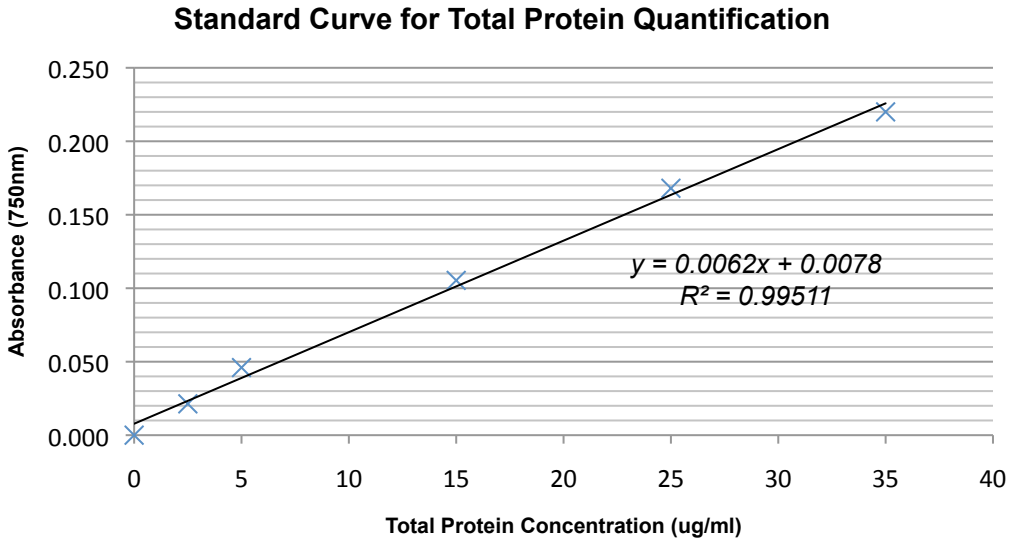
Annex 2: Standard curve used to quantify cellulose using varying cellulosic glucose concentrations



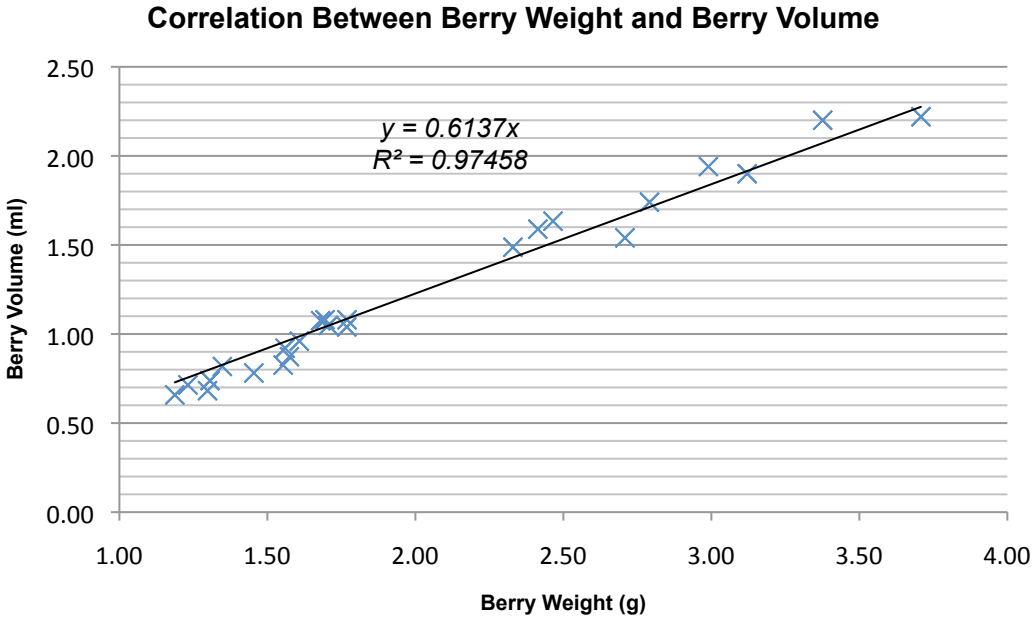
Annex 3: The standard curve used to quantify the total sugars present in the cell walls using varying glucose concentrations as a standard



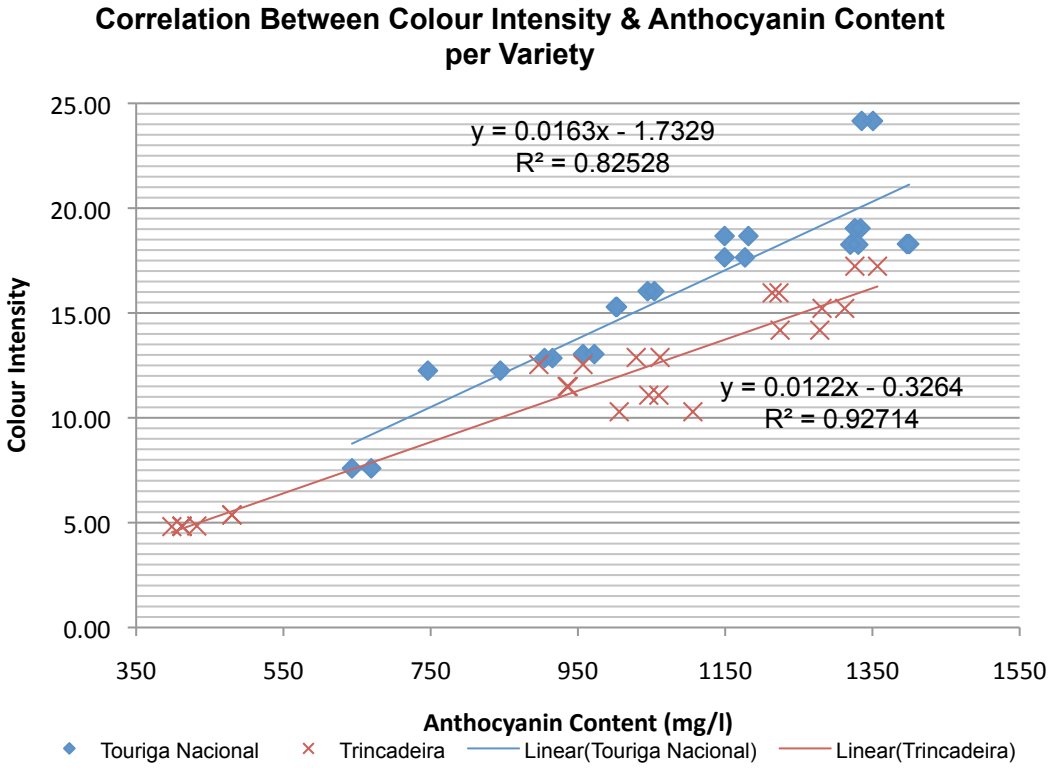
Annex 4: The standard curve used to quantify the total uronic acids present in the cell walls using varying galacturonic concentrations as a standard



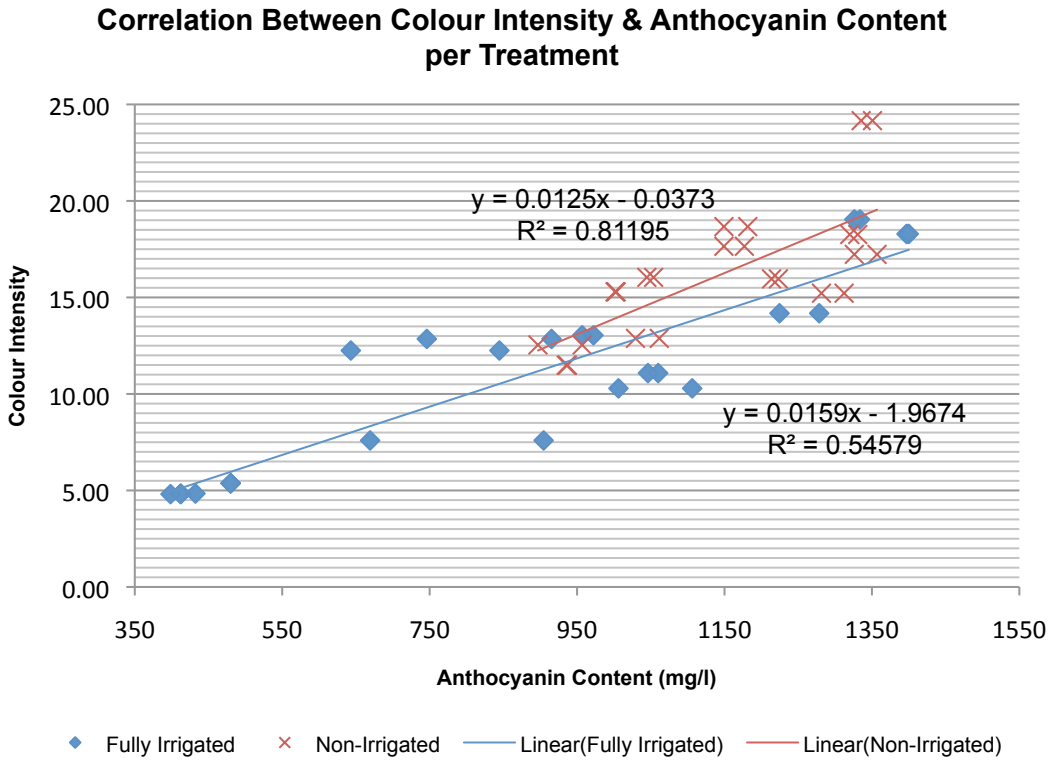
Annex 5: The standard curve used to quantify the total proteins present in the cell walls using varying bovine serum albumin (BSA) concentrations as a standard



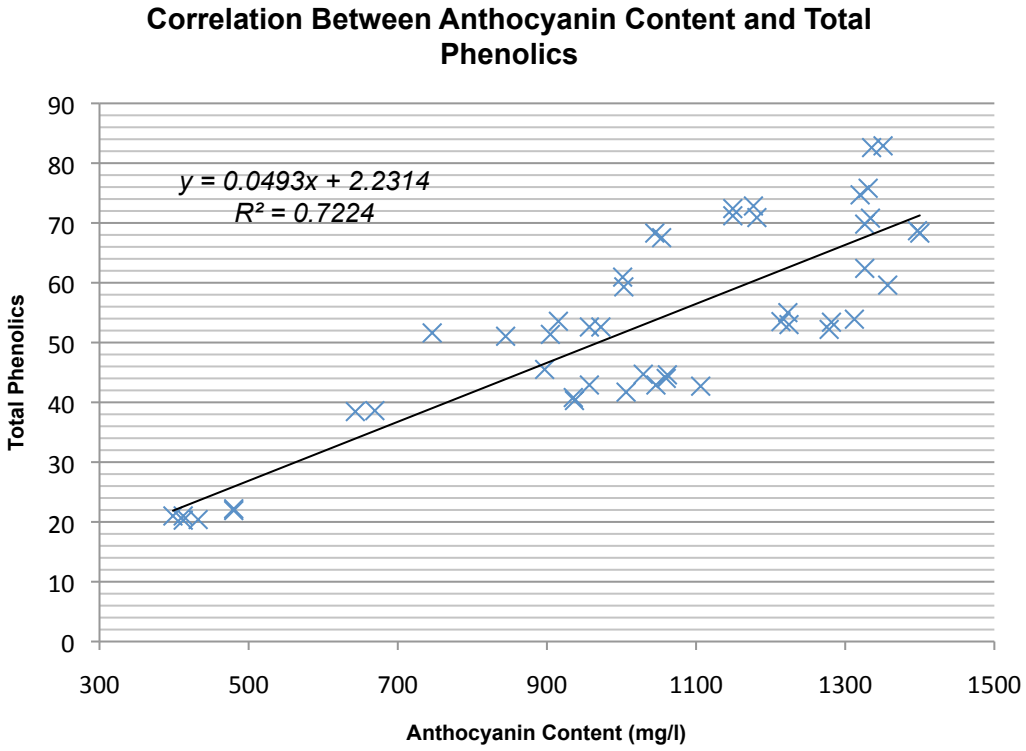
Annex 6: The correlation between Berry Volume (ml) and Berry Weight for Trincadeira and Touriga Nacional



Annex 7: The correlation between Colour Intensity and Total Anthocyanin Content (mg/l) per varieties of Trincadeira and Touriga Nacional, under both treatments of fully irrigated and non-irrigated.



Annex 8: The correlation between Colour Intensity and Total Anthocyanin Content (mg/l) per treatment of Fully Irrigated and Non Irrigated, under both varieties Trincadeira and Touriga Nacional.



Annex 9: The correlation between Total Phenols and Total Anthocyanin Content (mg/l) for Trincadeira and Touriga Nacional

	Touriga Nacional				Trincadeira			
	Non Irrigated		Fully Irrigated		Non Irrigated		Fully Irrigated	
Berry Weight (g/Berry)	1.26 ±0.07	*a*	1.66 ±0.05	*b*	1.34 ±0.11	*ac*	2.40 ±0.07	*d*
Berry Volume (ml/Berry)	0.69 ±0.04	*a*	1.04 ±0.07	*b*	0.77 ±0.05	*ac*	1.57 ±0.08	*d*
Brix	20.2 ±0.40	*a*	21.9 ±1.45	*ab*	24.0 ±0.95	*c*	22.5 ±1.18	*bc*
pH	3.64 ±0.01	*a*	3.75 ±0.05	*b*	3.86 ±0.04	*c*	3.86 ±0.03	*c*
Total Acidity (g/l of Ta)	4.17 ±0.21	*a*	3.57 ±0.65	*b*	4.17 ±0.21	*a*	3.53 ±0.25	*b*
Colour Intensity	16.33 ±1.21	*a*	10.90 ±2.88	*b*	12.30 ±0.72	*bc*	5.01 ±0.32	*d*
Anthocyanin Content (mg/l)	1071 ±74.5	*a*	787 ±118.2	*b*	969 ±62.8	*ac*	435.9 ±35.8	*d*
Total Phenolics	66.9 ±5.68	*a*	47.4 ±6.97	*b*	43.1 ±2.18	*bc*	21.3 ±0.77	*d*

Annex 10: The mean average of Berry composition (± Standard Error) at harvest for the two Touriga Nacional and Trincadeira, under Non Irrigated and Fully Irrigated conditions. The different letters signify means are significantly different within each parameter at P<0.001 according to Bonferroni t-test.

		Cellulose (μg/mg of Cell Wall)	*Total Sugar Content (μg/mg of Cell Wall)*			*Total Neutral Sugar Content (μg/mg of Cell Wall)*			*Total Uronic Acid Content (μg/mg of Cell Wall)*			*Total Protein Content (μg/mg of Cell Wall)*
			CDTA	0.1M KOH	6M KOH	CDTA	0.1M KOH	6M KOH	CDTA	0.1M KOH	6M KOH	
Touriga Nacional	*Non Irrigated*	129.0 ±17.7 *a*	10.1 ±2.5 *a*	172.5 ±10.9 *a*	96.1 ±4.8 *a*	1.79 ±1.01 *a*	23.5 ±5.65 *a*	37.0 ±3.77 *a*	12.4 ±4.12 *a*	201.9 ±14.5 *a*	47.2 ±5.15 *a*	8.10 ±1.35 *a*
	Fully Irrigated	144.5 ±20.0 *b*	8.52 ±2.3 *ab*	141.7 ±7.8 *b*	118.0 ±5.19 *b*	2.63 ±0.52 *a*	35.5 ±12.04 *b*	46.4 ±3.16 *b*	7.78 ±3.20 *b*	135.2 ±24.5 *b*	46.7 ±3.36 *a*	8.44 ±1.46 *a*
Trincadeira	*Non Irrigated*	179.3 ±31.9 *c*	10.6 ±6.2 *a*	176.5 ±6.4 *a*	118.0 ±6.2 *bc*	1.16 ±0.61 *a*	27.2 ±6.18 *ac*	45.8 ±4.69 *c*	13.7 ±3.05 *a*	191.6 ±10.9 *a*	51.2 ±3.37 *a*	8.14 ±1.26 *a*
	Fully Irrigated	249.8 ±41.7 *d*	6.46 ±1.04 *bc*	153.2 ±2.4 *b*	106.9 ±4.6 *d*	2.41 ±1.33 *a*	23.9 ±5.83 *cd*	42.4 ±4.04 *cb*	4.18 ±1.08 *b*	165.5 ±16.1 *c*	48.4 ±1.34 *a*	7.84 ±0.93 *a*

Annex 11.: The mean average of Berry composition (± Standard error) at harvest for the two Touriga Nacional and Trincadeira, under Non Irrigated and Fully Irrigated conditions. The different letters signify means are significantly different within each parameter at P<0.001 according to Bonferroni t-test