Carotenoid and chlorophyll content of *Vitis vinifera* cv. Merlot grapes during ripening with reference to variability in grapevine water status and vigour

By

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Previous research has shown that carotenoids are precursors of C_{13}-norisoprenoid aroma compounds in wine. C_{13}-norisoprenoids have low threshold values in wine with the most prominent C_{13}-norisoprenoids being β-damascanone and β-ionone which contribute honey and floral like aroma to wine. Chlorophyll and its derivates have also been detected in wine with potential to be precursors to aroma compounds.

Apart from the contribution of these pigments to wine aroma and quality they are vital role players in photosynthesis and are widely found in plants and plant products. The main functions of these pigments in plants are light collection and light-protection.

Research has shown that environmental conditions, climate, light exposure of bunches and soil water deficit influence the carotenoid content of grape berries. Furthermore the concentration of carotenoids and chlorophylls has also been shown to differ between cultivars. No research in this regard has been done on Merlot grape berries.

With this in mind, the aim of this study was to evaluate the effect of vigour and soil water content on the evolution of carotenoids and chlorophylls through ripening of grape berries from the cv. Merlot. However, when looking at methods to analyse carotenoids and chlorophylls in berry tissue, especially lyophilised tissue, there were no readily available methods. Thus, an extraction method to identify and quantify the carotenoid and chlorophyll profile of lyophilised tissue from unripe (green) to ripe (red) Merlot grape berries was needed. In this study the RP-HPLC method of Taylor *et al.* (2006) for carotenoids and the extraction method of Mendes-Pinto *et al.* (2004) were adapted to analyse both carotenoids and chlorophylls in lyophilised grape tissue. The RP-HPLC method baseline separated all the carotenoids and chlorophylls and their derivatives. Recovery of standards from mock extractions was high, indicating that the extraction procedure was acceptable. However, extraction recovery tested in the matrix of the grape tissue showed less promising results due to the high acid content of grape tissue. Violaxanthin, neoxanthin and the chlorophylls were especially sensitive to low pH conditions which facilitated their degradation. The degradation products of these compounds under acidic conditions were identified as pheophytin a, b, chlorophillide a, pyropheophytin b, *cis*-violaxanthin, *cis*-neoxanthin, neochrome, mutatoxanthin and luteoxanthin. There is a possibility that some degradation products were already present in the tissue due to lyophilisation (since the water in the berry was then removed and the acid concentrated). More work is needed to investigate the effect of lyophilisation and storage on the composition of grape tissue of different maturity. The extraction method for grape berry tissue at different ripening stages should also be optimised further to effectively neutralise tissue acidity, without compromising the extraction of carotenoids significantly, in especially green berry tissue. The question as to whether *cis*-isomers and chlorophyll degradation products are naturally present in grape berries or are formed during sampling and processing remains unanswered in the current study.

This study confirmed that in general carotenoids and chlorophylls decrease on a per berry (µg/berry) and concentration (µg/g) basis from veraison to harvest. Furthermore, this study was inconclusive in showing that vigour differences have an effect on the rate of synthesis/degradation of carotenoids, chlorophyll and some other ripening parameters, namely malic acid, total glucose and fructose, total tannin and total anthocyanin, from pre-veraison (pea size) to harvest. Additionally, no significant effect of soil water content on carotenoids, chlorophylls and ripeness parameters was found in this study, most likely due the fact that high soil water capacity was found in lower soil layers which may have prevented significant differences in grapevine water status. Experimental plots selected for vigour differences based on normalised difference vegetation index (NDVI) images, pruning mass and soil water measurements by means of a neutron probe, showed significant differences in soil water content in only the first 30 cm of the soil for the ripening seasons studied. Predawn plant water potential measurements, however, indicated that none of the experimental vines experienced severe water stress which was previously shown to effect carotenoid content of grapes.

The carotenoid 5,8-epoxy-β-carotene was quantified for the first time in grapes and represents a significant amount of the total carotenoids present at harvest. All the carotenoids and chlorophylls except β-carotene appeared to be sensitive to seasonal variation in climatic conditions. Lutein and β-carotene were found to be the most abundant carotenoids present in Merlot grape berries together with chlorophyll a for both seasons studied. The values of these carotenoids also correlated well with previous research. However, chlorophyll a was found in much larger quantities in Merlot berries compared to reported data. This is possibly because in this study the chlorophyll degradation products were included in the calculation of chlorophyll a.

Multivariate analysis showed promising preliminary prediction models (with correlation values of above 0.8 for both seasons analysed) for the prediction of the concentration of ripeness parameters (glucose, fructose, malic acid, total tannins and anthocyanins) with carotenoid and chlorophyll content. This result highlights the opportunity for the development of a rapid non-destructive method to measure carotenoids and chlorophylls in berries which in turn can predict optimal ripeness. Furthermore, since carotenoids are the precursors to C_{13}-norisoprenoid aroma compounds in wine a preview of the potential contribution of these aromas to wine might be evaluated. Further research is necessary to investigate the possibility of building and validating such models.

Opsomming

Vorige navorsing het getoon dat karotenoïede die voorlopers is van C_{13}-norisoprenoïed aromaverbindings in wyn. C_{13}-norisoprenoïede het lae drempelwaardes in wyn, met β-damassenoon en β-jonoon as die prominentste C_{13}-norisoprenoïede wat ‘n bydrae tot die heuning en blomagtige aroma van die wyn maak. Chlorofil en sy derivate is ook reeds in wyn bespeur, met die potensiaal om voorlopers van aromaverbindings te wees.

Buiten die bydrae van hierdie pigmente tot wynaroma en -kwaliteit is hulle ook belangrike rolspelers in fotosintese en kom hulle wydverspreid in plante en plantprodukte voor. Die vernaamste funksies van hierdie pigmente in plante is om lig te versamel en om as beskerming teen lig op te tree.

Navorsing het getoon dat omgewingstoestande, klimaat, ligblootstelling van die trosse en grondwatertekorte die karotenoïedinhoud van druiwekorrels beïnvloed. Verder is ook getoon dat die konsentrasie van karotenoïede en chlorofille tussen kultivars verskil. Geen navorsing is al in hierdie opsig op Merlot-druiwekorrels gedoen nie.

Met hierdie aspek in gedagte was die doelwit van hierdie studie om die effek van groeikrag en grondwaterinhoud op die evolusie van karotenoïede en chlorofille tydens die rypwording van druiwekorrels van die cv. Merlot te evalueer. Wanneer mens egter kyk na die metodes waarvolgens die karotenoïede en chlorofille in korrelweefsel geanaliseer word, is daar geen geredelik beskikbare metodes nie. ‘n Ekstraksiemetode om die karotenoïed- en chlorofilprofiel van geliofiliseerde weefsel van onryp (groen) tot ryp (rooi) Merlot-bessies te identifiseer en kwantifiseer was dus nodig. In hierdie studie is die RP-HPLC metode van Taylor *et al.* (2006) vir karotenoïede en die ekstraksiemetode van Mendes-Pinto *et al.* (2004) aangepas om beide karotenoïede en chlorofille in geliofiliseerde druiweweefsel te analiseer. Die basislyn van die RP-HPLC metode het all karotenoïede en chlorofille en hul derivate geskei. Herwinning van die standaarde vanaf skynekstraksies was hoog, wat aandui dat die ekstraksieprosedure aanvaarbaar was. Ekstraksieherwinning wat in die matriks van die druiweweefsel getoets is, het egter minder belowende resultate getoon as gevolg van die hoë suurinhoud van die druifweefsel. Violaxantien, neoxantien en die chlorofille was veral sensitief vir toestande van lae pH, wat hulle afbreking gefasiliteer het. Die afbrekingsprodukte van hierdie verbindings onder suurtoestande is geïdentifiseer as feofitien a en b, chlorofillied a, pirofeofitien b, *cis*-violaxantien, *cis*-neoxantien, neochroom, mutatoxantien en luteoxantien. Daar is ‘n moontlikheid dat sommige afbreekprodukte reeds in die weefsel teenwoordig was as gevolg van liofilisering (aangesien die water in die korrel reeds verwyder was en die suur gekonsentreerd was). Meer werk is nodig om die effek van liofilisering en berging op die samestelling van druifweefsel van verskillende rypheid te bepaal. Die ekstraksiemetode vir druifkorrelweefsel op verskillende

stadia van rypwording moet ook verder geoptimaliseer word om weefselsuurheid doeltreffend te neutraliseer, sonder om die ekstraksie van karotenoïede noemenswaardig te kompromitteer, veral in groen korrelweefsel. Die vraag of *cis*-isomere en chlorofil afbreekprodukte natuurlik in die druifkorrels teenwoordig is en of hulle tydens monsterneming en prosessering gevorm word, kon nie in hierdie studie beantwoord word nie.

Hierdie studie het bevestig dat karotenoïede en chlorofille oor die algemeen op ‘n korrel (µg/korrel) en konsentrasie (µg/g) basis afneem vanaf deurslaan tot oes. Hierdie studie het nie daarin geslaag om te toon dat groeikragverskille vanaf voor-deurslaan (ertjiekorrelgrootte) tot oes ‘n effek het op die tempo van sintese/afbreking van karotenoïede, chlorofil en ander rypwordingsparameters nie, naamlik op appelsuur, totale glukose en fruktose, totale tannien en totale antosianien. Daar is ook in hierdie studie geen noemenswaardige effek van grondwaterinhoud op karotenoïede, chlorofille en rypheidsparameters gevind nie, heel moontlik as gevolg van die feit dat hoë grondwaterkapasiteit in die laer grondlae gevind is, wat betekenisvolle verskille in wingerdwaterstatus kon verhoed het. Eksperimentele persele wat gekies is vir groeikragverskille op grond van genormaliseerde verskil plantegroei indeks (NDVI) beelde, snoeimassa en grondwatermetings met ‘n neutronvogmeter het net in die eerste 30 cm van die grond noemenswaardige verskille in grondwaterinhoud getoon vir die rypwordingseisoene wat bestudeer is. Voor-sonopkoms plantwaterpotensiaalmetings het egter aangedui dat geen van die eksperimentele wingerdstokke ernstige waterstres ervaar het nie. Sulke stres is voorheen aangedui om ‘n effek op die karotenoïedinhoud van druiwe te hê.

Die karotenoïed 5,8-epoksi-β-karoteen is vir die eerste keer in druiwe gekwantifiseer en verteenwoordig ‘n noemenswaardige hoeveelheid van die totale karotenoïede wat met oes teenwoordig is. Al die karotenoïede en chlorofille behalwe β-karoteen blyk sensitief vir seisoenale verskille in klimaatstoestande te wees. Luteïen en β-karoteen was die volopste karotenoïede in die Merlot-druifkorrels, tesame met chlorofil a, vir beide seisoene wat bestudeer is. Die waardes van hierdie karotenoïede was ook goed gekorreleer met vorige navorsing. Chlorofil a is egter in baie groter hoeveelhede in Merlot-korrels gevind in vergelyking met dít wat in die data gerapporteer is. Die rede hiervoor is moontlik dat die chlorofil-afbreekprodukte in hierdie studie in die berekening van chlorofil a ingesluit is.

Meerveranderlikeontleding het belowende voorlopige voorspellingsmodelle getoon (met korrelasiewaardes van meer as 0.8 vir beide die seisoene wat geanaliseer is) vir die voorspelling van die konsentrasie van rypheidsparameters (glukose, fruktose, appelsuur, totale tanniene en antosianiene) met karotenoïed- en chlorofilinhoud. Hierdie resultaat beklemtoon die geleentheid vir die ontwikkeling van ‘n vinnige, nie-destruktiewe metode om karotenoïede en chlorofille in korrels te meet, wat op sy beurt optimate rypheid kan voorspel. Aangesien karotenoïede die voorlopers van C_{13}-norisoprenoïed aromaverbindings in wyn is, kan ‘n voorskou van die potensiële bydrae van hierdie aromas tot wyn moontlik verder evalueer word.

Verdere navorsing is nodig om die moontlikheid van die bou en geldigheidsbepaling van sulke modelle te ondersoek.

This thesis is dedicated to Stellenbosch and all its happy student memories

Biographical sketch

Zindi Kamffer was born in Evander on 1 December 1984. She matriculated at Duineveld High School in Upington in 2002. Zindi enrolled at Stellenbosch University in 2003 and obtained the degree BScAgric in Viticulture and Oenology in December of 2006. In 2007 she enrolled for the degree MScAgric in Viticulture, also at the Stellenbosch University.

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Preface

This thesis is presented as a compilation of five chapters and appendixes. Each chapter is introduced separately, with the results presented in chapters' three to four and concluded in chapter five.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
A review of the viticultural control of carotenoids and chlorophyll biochemistry in grape berry ripening

Chapter 3 **Technical report**
Optimization of a method for the extraction and quantification of chlorophyll and carotenoids in grape berries (*Vitis vinifera*) cv. Merlot

Chapter 4 **Research results**
Influence of grapevine vigour and soil moister on the carotenoid profile of cv. Merlot grape berries

Chapter 5 **General discussion and conclusions**

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Chapter 1

General Introduction and Project aims

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

In recent years increasing attention in viticultural research has been given to grape berry carotenoids since they have been identified as potential precursors to a group of potent aroma compounds in wine, the C_{13}-norisoprenoids (Baumes *et al.* 2002). The C_{13}-norisoprenoids can make a positive contribution to the complexity and quality of wine, contributing their floral and honey like notes to wine aroma (Kanasawud and Cruzet 1990; Kovats 1987; Ohloff 1978). In order to optimise the concentration of these compounds in grapes the viticultural factors which influence their metabolism in grape berries needs to be better understood.

Carotenoids in unripe grape berries function as light-harvesters and quenchers of excess light in the photosynthetic systems of the chloroplast together with chlorophyll (Van den Berg *et al.* 2000; Krinsky 1979). It has been shown that variation in the level of light incident on a grape cluster may have an effect on berry carotenoids in experiments comparing sun-exposed and shaded grape bunches (Razungles *et al.* 1998; Bureau *et al.* 1998; Bindon 2004; Bureau *et al*. 2000; Oliveira *et al*. 2004). There is also evidence that vigorously growing grapevines with denser canopies may have altered light and temperature conditions of the bunch zone, or canopy microclimate. This, in turn, might directly or indirectly affect carotenoid synthesis and breakdown. As yet, no research has given clear direction to this question. Apart from the effect of sunlight on the carotenoid composition of grapes, research has not thoroughly addressed the effects of other environmental conditions or vine management practices on the grape carotenoid profile. It has been speculated that vine water deficit might directly or indirectly affect the carotenoid content of grapes since some studies have shown that water deficit in grapevines can elevate the level of carotenoids in grapes (Oliveira *et al.* 2004; Bindon *et al.* 2007). This is conceivable, since the plant hormone abscisic acid (ABA), which controls both stress signalling and regulates ripening in grapevines is closely related to the carotenoid metabolic pathway (Cutler and Krochko 1999; Liotenberg 1999; Taylor *et al.* 2000; Antolin 2003).

Analysis of carotenoids and chlorophylls is not an easy task since they are susceptible to degradation and structural alteration in the presence of acids, heat treatment and exposure to light (Rodriguez-Amaya *et al.* 2008; Van den Berg *et al.* 2000; Oliver and Palou *et al.* 2000). Methods available for analysing carotenoids and chlorophylls are time consuming and relatively expensive, involving both spectroscopic and chromatographic methods. Various high-performance-liquid-chromatography (HPLC) techniques have been used for the identification and quantification of grape carotenoids (Bindon 2004; Oliveira *et al.* 2004; Steel and Keller 2000) but as yet, no method has reported the simultaneous measurement of carotenoids and chlorophylls. The development of a robust analytical method is important for a number of reasons. Since carotenoids and chlorophylls may serve as potential ripeness indicators, as well

as markers for wine quality (aroma and phenolic potential), the development of a rapid and non-destructive technique for the measurement of these pigments *in situ* (vineyard) could be a valuable tool for grape and wine producers. Non-destructive assessment of chlorophyll, carotenoid and anthocyanin content in higher plant leaves has been studied by Gitelson *et al.* (2002) whereby the relationship between reflectance and pigment content were established and quantitative techniques for pigment estimation in various leaf species with diverse pigment content and composition were developed. Chlorophyll fluorescence measurements have been found to be well-suited to non-invasively determine sugar accumulation in white grape berries cv. Bacchus and Silvaner (Kolb *et al*. 2006). The assessment of anthocyanins in whole grape bunches via chlorophyll fluorescence imaging has also been developed by Agati *et al.* (2008), allowing for the non-invasive assessment of phenolic maturity in the vineyard. Other technologies exist which may allow for the prediction of pigments in grapes, namely NIR spectroscopy, which has the added advantage of being transportable.

However, before any such rapid techniques can be implemented, a robust, validated analytical method is necessary, such as RP-HPLC analysis. Furthermore, the validity of taking a non-destructive approach needs to be evaluated by application to a vineyard scenario, over multiple seasons. By this approach, the potential relationships of fluorescent pigments to other more traditionally used ripening parameters for grape maturity can be evaluated. Following this, the relevance of using pigments such as chlorophylls and carotenoids as indicators of 1) grape ripeness relative to other ripeness parameters; and 2) vineyard variability, can be determined.

This study has undertaken the approach of developing a method to accurately and reproducibly quantify the content of carotenoids and chlorophylls in grape berries using cv. Merlot as an example. Additional to this, the potential effects of grapevine vigour and soil moisture on grape carotenoid and chlorophyll levels are explored during berry development within a single vineyard over two seasons. Preliminary work on the relationship between berry ripeness parameters such as total grape anthocyanins, tannin, malic acid, and total sugars with the profile of chlorophylls and carotenoids in grape berries has been done using multivariate statistics, and will be discussed.

1.2 SPECIFIC PROJECT AIMS

This study aimed to explore the changes in the carotenoid and chlorophyll content of grape berries during ripening, using field measurements of grapevine vigour and soil moisture in order to observe differences in the pigment profile in response to these factors, if any. As a prerequisite to this, a method was developed for the simultaneous extraction and quantification of carotenoids and chlorophylls in Merlot grape berries, using RP-HPLC. Furthermore, the study aimed to explore the relationship between carotenoid and chlorophyll content and traditional grape berry ripeness parameters such as total sugar, malic acid, anthocyanin and tannin content, determined per berry.

These goals were achieved using the following objectives:

i) To optimise an extraction method for both carotenoids and chlorophylls in lyophilised grape berry tissue.

ii) To explore the changes in the content of carotenoids and chlorophylls during grape ripening from pre-veraison to harvest by quantifying these pigments at different ripening stages via RP-HPLC.

iii) To quantify grapevine responses to differences in vigour and soil moisture in terms of pruning weight, bunch exposure, shoot growth, leaf water potential and neutron probe measurements, and explore the relationship between these, and iii), if any, using multivariate analytical techniques (PCA).

iv) To explore the potential relationship, if any, on carotenoid and chlorophyll content on grapes to standard measures of grape 'ripeness', namely malic acid, total sugar (glucose and fructose) and anthocyanin content of grapes, using Merlot berries from a single vineyard as the study sample.

v) To build prediction models with chemometric software to explore the potential prediction of ripening parameters total glucose and fructose, total tannins, total anthocyanin and malic acid per berry fresh weight from carotenoid and chlorophyll content per berry fresh weight using multivariate analysis (PLS2).

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Chapter 2

Literature review

A review of carotenoid and chlorophyll biochemistry in grape berry ripening with reference to its significance for viticulture

2.1 INTRODUCTION

Wine aroma is one of the most important aspects of wine quality since it contributes to the first perception of the wine consumer. Viticultural practices to improve aroma in order to make better, more strongly preferred and higher quality wine is an important research field for the grape and wine industry. Aroma in wine is formed and manipulated at many stages of the wine production process: it is influenced by the aroma formed by yeast, aromas extracted from oak and the aromas derived from precursors in the grape itself. Aromas originating from the grape berries make a large contribution to the aroma and complexity of the final product. This is especially evident in the case of cultivar wines where the cultivar-specific aroma or precursor originates in the grapes of a particular variety (genotype). Thus, to study the effect of viticultural practices on the profile of precursor compounds to aroma is of utmost importance, particularly when considering the optimal production of aroma-rich, cultivar-specific wines.

It is currently thought that carotenoids make an important contribution with regards to grape-derived wine aroma, especially to the typical aroma of some cultivars (Ferreira *et al.* 2008). Sefton *et al*. (1993) studied the volatile composition of cv. Chardonnay and identified 108 compounds from which more than 70% of the total concentration of volatile secondary metabolites comprised C_{13}-norisoprenoids. Research has shown that carotenoids are the likely precursors to C_{13}-norisoprenoids which is a very significant group of aroma compounds in wine because they have low olfactory threshold values (Etievant *et al.* 1991). Chlorophylls and their derivatives are also reported to be found in wine (De Pinho *et al.* 2001) and have potential in being precursors to aroma compounds (Sefton *et al.* 1993).

As a class of compounds, carotenoids are closely related to chlorophyll with regards to their function in photosynthesis but are structurally different . Carotenoids act as accessory pigments in light-harvesting antennae by transferring energy to the photosystem reaction centres and also acting as quenchers of triplet excited states in chlorophyll molecules generated during photosynthesis (Demmig-Adams *et al.* 1996).

One of the important C_{13}-norisoprenoids which contributes to wine aroma is β-ionone with a low threshold value of 90 ng/L (in a model base wine) (Kotseridis *et al.* 1999b). β-ionone has a violet like aroma and can be formed as a cleavage product of the carotenoid β-carotene (Kanasawud and Crouzet 1990) and zeaxanthin, a xanthophyll (Mathieu *et al.* 2005). β-damascenone is another C_{13}-norisoprenoid found in wine, with a threshold value of 50 ng/L in 10% alcohol (Guth 1997), its aroma notes have been described as honey-like

(Kovats 1987), flowery and ionone-like (Ohloff 1978). Recently it was demonstrated that β-damascanone can be formed directly from the carotenoid neoxanthin (Bezman *et al.* 2005). Other examples of C_{13}-norisoprenoids found in wine are, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and vitispirane (Oliveira *et al.* 2006). As a point of interest, carotenoids have potential medical benefits for humans in preventing cancer and cardiovascular related diseases (Cooper 2004; Krinsky and Johnson, 2005).

In this chapter, the chemical structure, biosynthesis, degradation and major roles of carotenoids and chlorophyll in grapes are discussed, with specific reference to changes occurring through grape maturation. Secondly, research that has been done on the viticultural control of the carotenoid and chlorophyll profile in relation to other important compounds through ripening in grape berries will follow. The interactive effects of sunlight, terroir, cultivar selection, soil type and water stress will be discussed. Carotenoid biochemistry, however, will be the main focus of this literature review in order to gain a better understanding of above-mentioned impacting factors.

2.2 LOCATION, ROLE AND STRUCTURE OF CAROTENOIDS AND CHOROPHYLL

2.2.1 LOCATION OF CAROTENOIDS AND CHLOROPHYLLS IN HIGHER PLANTS AND GRAPE BERRIES

In fruit and flowers, carotenoids are located in chromoplasts (Goodwin 1980). A chromoplast is a plastid located in plant cells where carotenoids are synthesised and stored (Deli *et al.*1992; Minguez-Mosquera *et al.* 1994). However, carotenoids are also present in plastids called chloroplasts (Figure 2.1). Work by Camara and Moneger (1978) confirmed that carotenoids and chlorophylls are synthesised in chloroplasts but carotenoids are additionally synthesised in chromoplasts (Britten 1979; Deli *et al.* 1992; Minguez-Mosquera *et al.* 1994*;* Rabinowitch *et al.* 1975).

It has been found that carotenoids located in the chloroplast are synthesised as part of an integrated system which underpins processes associated with plastid development (Goodwin 1993). An etioplast is a chloroplast which has not been exposed to light. During light-dependent transitions of etioplasts to chloroplasts, massive structural and biochemical modifications appear as well as pigment (carotenoids and chlorophylls) changes (Van den Berg *et al.* 2000).

In maize leaves more carotenoids have been found in mature chloroplasts than in etioplasts and the conversion of etioplasts to chloroplasts by light stimulates the synthesis of carotenoids in parallel to the biosynthesis of chlorophyll (Albrecht and Sandmann 1994).

It is evident that the regulation of carotenoids is linked to the development and transformation of plastids, and that light is key in regulating this process.

Plastids continue to divide in developing tissues that are no longer meristematic. Many early studies demonstrated that the plastid number per cell varies considerably depending on the cell type, developmental stage, and environmental conditions which the cells are subjected to (Boffey and Lloyd 1988; Pyke 1999). In spite of these observations, little is understood about how plastid number per cell is controlled at the molecular level, or how it is related to the rate of plastid division (Boffey and Lloyd 1988; Pyke 1999). Neither chromoplasts nor etioplasts have been reported in *Vitis vinifera* (Hardie *et al*. 1996). Thus it appears that carotenoid and chlorophyll synthesis and breakdown in the grape berry is primarily located in the chloroplast. Chlorophylls and carotenoids are situated in the thylakoid membranes within the chloroplast (Figure 2.1).

Carotenoids are bound mostly to specific chlorophyll/carotenoid-binding protein complexes of the two photo systems, namely photosystem I and photosystem II (PSI and PSII) (Yamamoto and Bassi 1996). Between PSI, PSII and among the different protein complexes, carotenoids are unevenly distributed. Furthermore within each photosystem carotenoids are also unevenly distributed, with PSI enriched in β-carotene and PSII enriched in lutein. In PSII, most of the carotenoid β-carotene is present in the core complexes closely surrounding the reaction centre. The rest of the carotenoids present are in the remaining light-harvesting antennae that are made up of several functional components (Demmig-Adams *et al.* 1996). In the chloroplast, the carotenoids are masked by the presence of chlorophylls. The pattern of chloroplast carotenoids has been found to be universally uniform and contains four basic carotenoids, namely one carotene and three xanthophylls. Additional minor pigments like α-carotene, α- and β-cryptoxanthin, isolutein (lutein 5,6-epoxide), zeaxanthin and antheraxanthin (zeaxanthin 5,6-epoxide) were also found. The approximate levels of chloroplast carotenoids found were as follows: lutein, which predominates, 40-57%; β-carotene 25-40%; violaxanthin 9-20%; and neoxanthin 5-15% (Gross 1991).

In grapes, the total level of carotenoids in chloroplasts declines from veraison onward with a significant reduction when the colour, size and texture of the berries change (Razungles *et al.* 1987). This decline of carotenoids from the time of veraison corresponds with the disappearance of chlorophyll in the chloroplast. The decline of carotenoids may potentially be part of the catabolism pathway of chlorophyll since both molecules form part of the photosynthetic apparatus in the chloroplasts, which are not functional without the presence of chlorophyll (Hardie *et al.* 1996).

In grape berries, it has been found that grape skins contribute approximately 65% of carotenoids (lutein, monoesters of xanthophylls and β-carotene) while the contribution of the pulp is only 35% (De Pinho *et al*. 2001). In grapes, the content of neoxanthin was found to be three times higher in skin than in pulp, as was the proportion of β-carotene to total carotenoids in these tissues (De Pinho *et al*. 2001). Levels of lutein and monoesters of xanthophylls are evenly distributed between skin and pulp. Razungles *et al.* (1988) in his study on Muscat berries also reported higher amounts of carotenoids in berry skins than in pulp with carotenoids found to be absent in juice. Razungles *et al.* (1988) suggested that carotenoids are highest in the skin since photosynthetic activity is higher in skins than in pulp, and will be associated with a similar distribution in chlorophyll content.

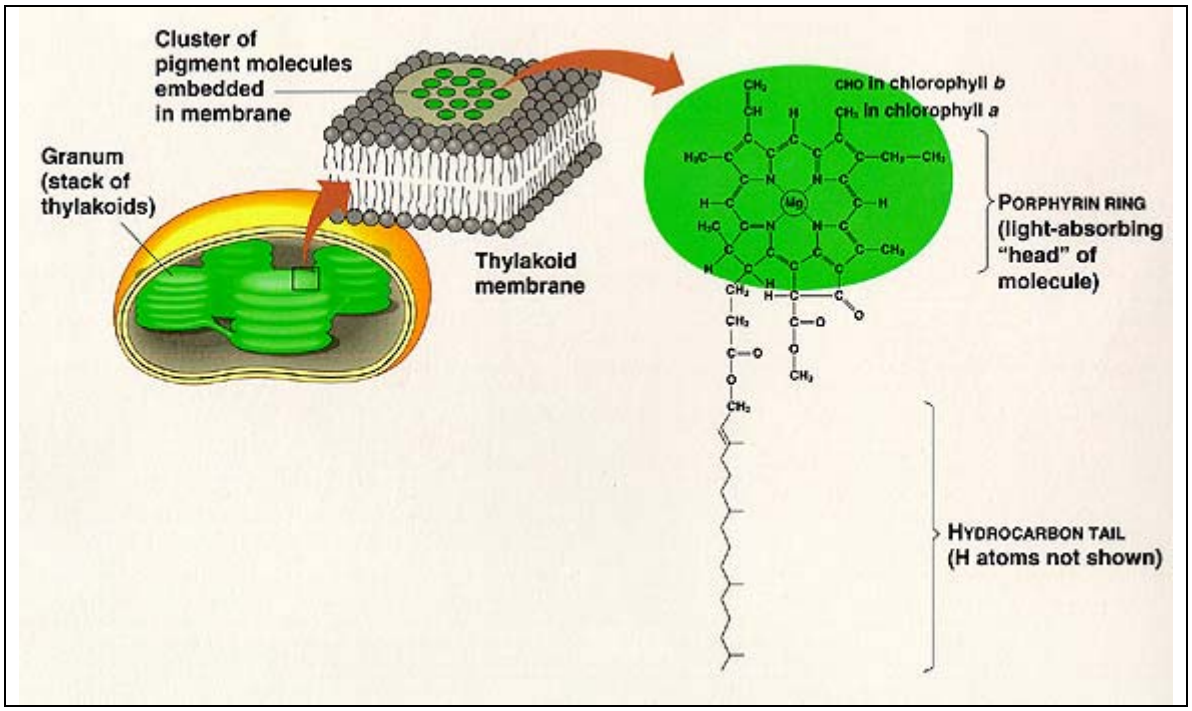

Figure 2.1 The ultrastructure of a chloroplast showing the location of carotenoids and chlorophylls within the chloroplast (adapted from http://fig.cox.miami.edu/Faculty/Dana/chlorophyll.jpg)

2.2.2 THE ROLE OF CAROTENOIDS AND CHLOROPHYLLS IN HIGHER PLANTS AND GRAPE BERRIES

Carotenoids are associated with multi-protein complexes of plant chloroplast membranes which makes up the photosynthetic systems (PS I and PS II). In these complexes the two main functions of carotenoids in photosynthesis are photo-protection and light harvesting. Both these functions involve an interaction with chlorophyll, but represent different directions in terms of energy transference. Photo-protection is the channelling of photochemical energy away from chlorophyll whereas light harvesting is the collection of light energy and its subsequent transfer to chlorophyll in photochemical form (Krinsky

1979). Carotenoids are essential to higher plants as photo-protectors since the transfer of energy to chlorophylls converts them to a higher energy (excited) state. These excited molecules can cause some lethal mutations in carotenoid synthesis which could damage the photosynthetic apparatus (Van den Berg *et al.* 2000; Krinsky 1979).

During photosynthesis the xanthophylls lutein, violaxanthin, neoxanthin and to a lesser extent β-carotene operate as accessory light harvesting pigments. The xanthophyll, zeaxanthin is primarily responsible for the safe dissipation of excess light energy as heat via the xanthophyll cycle whereas β-carotene is a potent antioxidant amongst others (Packer and Douce 1987).

In grape berries the physiological role of carotenoids in photosynthesis has not been widely studied. Potentially, they play the same role as in leaves in the early stages of fruit development and ripening by harvesting light and protecting the photosynthetic apparatus against excess sunlight energy. However, it is well known that carotenoids are the precursors to some grape aroma compounds (C_{13}-norisoprenoids) (Baumes *et al.* 2002) and absicic acid, formed via the carotenoid biosynthetic pathway (Marin *et al.* 1996) is a hormonal signal controlling the onset of berry ripening (Coombe and Hale 1973).

Chlorophyll is the main photoreceptor in photosynthesis, the light-driven process in which carbon dioxide is fixed to yield carbohydrates and oxygen (Quach *et al.* 2004). Limited data is available on the antioxidant capacity of chlorophyll (Buratti *et al.* 2001). More data on the antioxidant capacity of chlorophyll in grapes could add value to grape products in regards to their health benefits (Razungles *et al*. 1996; Endo 1985).

2.2.3 STRUCTURE OF CAROTENOID AND CHLOROPHYLL MOLECULES

Carotenoid structure consists of a system of long, aliphatic conjugated double bonds which are responsible for the various physical, biochemical and chemical properties they impart to the molecule (Van den Berg *et al.* 2000). These extended systems of conjugated bonds designate carotenoids as a group of deeply red or yellow pigments with absorption maxima of between 400 and 500 nm, the range of which is dependent upon the amount of conjugated double bonds per molecule (Van den Berg *et al.* 2000). The carbon-carbon double bonds can exist in the *cis*- or *trans*- isomer configurations depending on the arrangement of substitutes (Weedon and Moss 1995; Zechmeister and Polgar 1943). In natural sources, carotenoids occur mainly in the all-trans configuration (Chandler and Schwartz 1987). Isomerisation of all-*trans*-carotenoids to *cis*-isomers is promoted by contact with acids, heat treatment and exposure to light (Rodriguez-Amaya *et al.* 2008; Van den Berg *et al.* 2000; Oliver and Palou 2000). Additionally, these alterations can affect

the configuration and structure of these lipophilic pigments. Most of the carotenoids are composed of eight isoprene units with the molecular formula $C_{40}H_{56}$ (Armstrong and Hearst 1996).

There are two classes of carotenoids based on their structure, namely carotenes and xanthophylls (Figure 2.2). Oxygenated carotenes are called xanthophylls and can have various combinations of e.g. hydroxyl-, epoxy-, alcohol-, aldehyde-, keto-, lactone-, carboxylic acid-, ester or phenolic functional groups (Felt *et al.* 2005). In mature grapes the most common carotenes are β-carotene and lutein, representing almost 85% of the total. They are accompanied by minor xanthophylls such as neoxanthin, violaxanthin, lutein-5,6-epoxide, zeaxanthin, neochrome, flavoxanthin and luteoxanthin (Baumes *et al.* 2002). Most of the carotenoids reported to be found in berries are in the *trans*-configuration. However *cis*-isomers of lutein, β-carotene and neoxanthin have been reported by Mendes-Pinto *et al.* (2004, 2005). It is not certain if these isomers do exist in grape berries or if it is an artefact of sample processing.

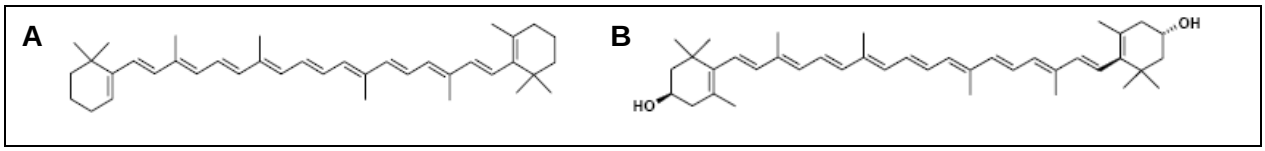

Figure 2.2 The structure of **A:** β-carotene an example of the carotene group. **B:** Zeaxanthin an example of the xanthophylls group of carotenoids (Van den Berg *et al.* 2000).

Chlorophyll is a cyclic tetrapyrolle with a structure similar to the heme group of globins (hemoglobin, myglobin) and cytochromes (Figure 2.3). The central metal ion in chlorophyll is magnesium. Although several types of chlorophyll exist, chlorophyll a is the major pigment and chlorophyll b is accessory pigments which exist in a ratio of approximately 3 to 1 in higher plants (Gross 1991). The difference between chlorophyll a and chlorophyll b is a methyl side-chain in chlorophyll a which is substituted with a formyl group in chlorophyll b (Gross 1991). Chlorophylls are green in colour because they absorb strongly in the red and blue regions of the visible spectrum. Small differences in the structures of the two chlorophylls produce differences in the absorption maxima of chlorophyll a and chlorophyll b.

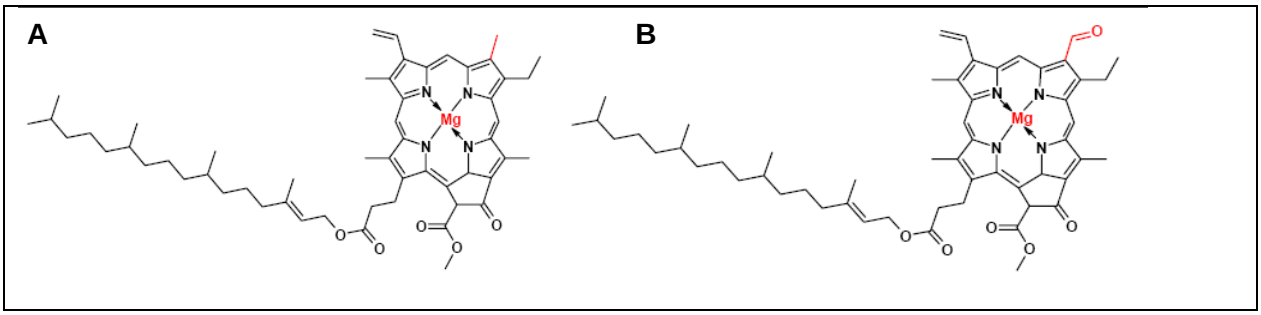

Figure 2.3 The structure of **A:** chlorophyll a and **B:** chlorophyll b (adapted from Schoefs (2002))

2.3 BIOSYNTHESIS AND DEGRADATION OF CAROTENOIDS AND CHLOROPHYLLS

2.3.1 BIOSYNTHESIS AND DEGRADATION OF CAROTENOIDS

The biosynthesis of carotenoids follows the non-melavonate pathway (Britten 1979) via isopentyl diphosphate (IPP) as a precursor, obtained by condensation of pyruvate and glyceraldehyde-3-phophate via 1-deoxy-D-xylulose-5-phophate (Figure 2.3) (Lichtenthaler *et al.* 1997). According to research it is not yet certain whether the plastids can synthesise carotenoids directly from isopentyl diphosphate (IPP) or whether IPP is imported to the plastid (chloroplast) but it does appear that the site of synthesis of the early precursors depends upon the developmental stage of the chloroplast (Goodwin 1993). Furthermore Britton *et al.* (1982) reported that biogenesis of carotenoids takes place in the chloroplast and are an integral part of the chloroplast development. Carotenoid synthesis is also closely linked to biosynthesis of other chloroplast components like pigment complexes, lipids and other material which forms part of the thylakoid membranes. If one component is not available, the entire chloroplast construction is disrupted. Gross (1991) discussed carotenoid biosynthesis involving six stages namely: i) formation of mevalonic acid ii) formation of geranylgeranyl pyrophosphate iii) formation of phytoene iv) desaturation of phytoene v) cyclization vi) formation of xanthopylls.

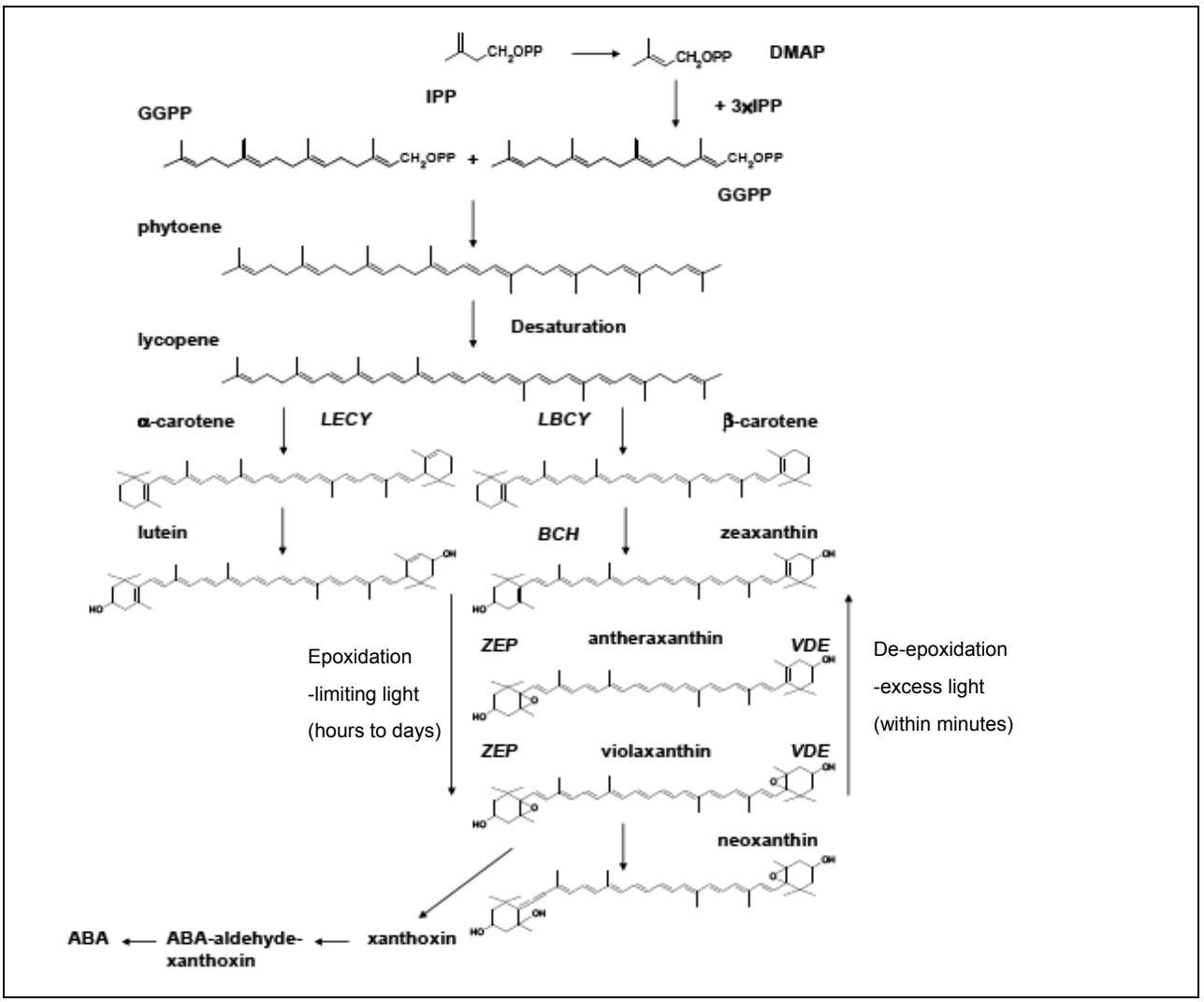

Figure 2.4 A simplified diagram of the carotenoid biosynthesis pathway in plants. LECY, lycopene ε-cyclase; LBCY, lycopene β-cyclase; BCH, β-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; ABA, abscisic acid (Hirschberg 2001).

The last three steps in Figure 2.4 are known as the xanthophyll cycle and entail the de-epoxidation and epoxidation interconversions of three xanthophylls: zeaxanthin, antheraxanthin and violaxanthin (Yamamoto and Bassi 1996). These interconversions are catalysed by two enzymes, zeaxanthin epoxidase and violaxanthin de-epoxidase that are localized on opposite sides of the thylakoid membrane. Enzymes involved in the biosynthesis of carotenoids are difficult to study since they are membrane-associated or integrated into membranes which make them difficult to isolate (Sandmann 1994). The xanthophyll cycle is involved in the quenching of excess photon energy and the conversion of these specific xanthophylls is therefore light-dependent and light-regulated (Demmig-Adams et al. 1995).

Furthermore Demmig-Adams *et al.* (1996) reviewed the time scale in which reactions in the xanthophyll cycle takes place and it varies from a few minutes (de-epoxidation) to hours (epoxidation) in response to various environmental conditions (Adams *et al.* 1995).

Changes in the pH within the thylakoid membrane facilitate the typical biochemical conversions in the xanthophyll cycle over the course of minutes up to a day. Variation in seasonal and weather conditions come in to play during periods when the rates of photosynthesis, and thus the rates of utilization of absorbed light, are low throughout the day e.g. in cloudy winter periods (Demmig-Adams *et al.* 1996).

Little information is available about the turnover of carotenoids within the xanthophyll cycle. Several oxidative cleavage reactions of carotenoids are known. Scission of epoxy-carotenoids such as violaxanthin and neoxanthin initiates the synthesis of abscisic acid (ABA). The abscisic acid-deficient mutant aba2 of *Nicotiana plumbaginifolia* is blocked in the epoxidation reaction of zeaxanthin to violaxanthin indicating that carotenoid precursors are essential for ABA biosynthesis (Nussaume *et al.* 1996).

2.3.2 BIOSYNTHESIS AND DEGRADATION OF CHLOROPHYLLS

Chlorophyll is formed as part of a network of pathways which forms various tetrapyrroles and can be subdivided into three parts, i) formation of 5-aminolevulinic acid (ALA), the committed step for all tetrapyrroles, ii) formation of protoporphyrin IX (Proto) from eight molecules of ALA and iii) formation of chlorophyll in the magnesium branch (Figure 2.5) (Eckhardt *et al.* 2004). The enzymes which contribute to chlorophyll synthesis correspond to the biochemical nature of the metabolic intermediates. The early steps in chlorophyll synthesis are catalysed by highly soluble enzymes which are located mostly in the chloroplast stroma. The later steps are associated with thylakoid or inner envelope membranes of the chloroplast (Eckhardt *et al.* 2004).

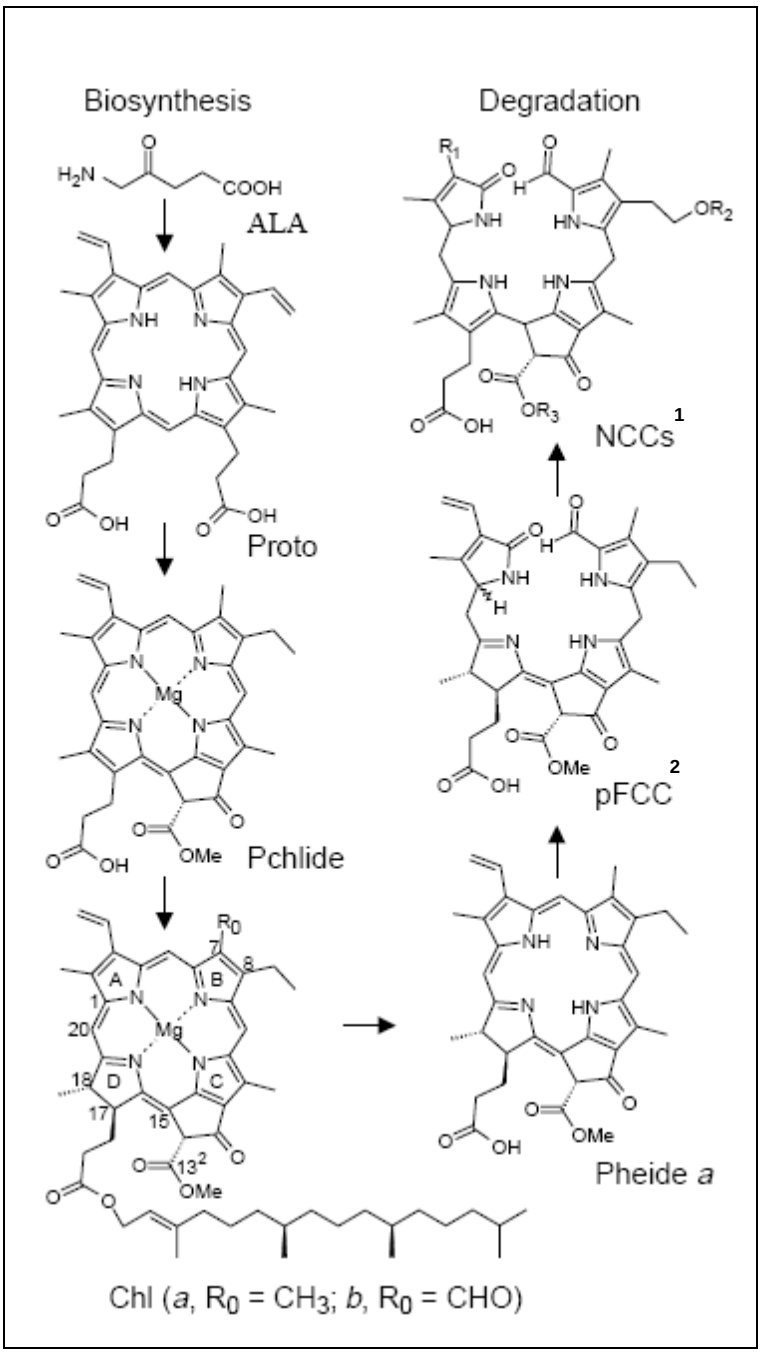

Figure 2.5 Structures of important intermediates of chlorophyll biosynthesis and degradation. ALA (5-aminolevulinic acid); Proto (protoporphyrin); Pchlide (protochlorophyllide); NCCs[1] (non-fluorescent chlorophyll catabolite); pFCC[1] (primary fluorescent chlorophyll catabolite); Pheide a (pheophorbide a) (adapted from Eckhardt *et al.* 2004).

Chloropigments are susceptible to degradation either by chemical or enzymatic means. Chemical degradation occurs in response to weak acids, oxygen, light and heat and can lead to the formation of a large number of degradation products. Pheophytinization, epimerization, and pyrollysis, of chlorophyll can occur, but if light is implicated hydroxylation, oxidation or photo-oxidation, are the major chemical degradation routes (Gross 1991). Chlorophyll a can readily be converted to pheophytin a by adding a weak or diluted acid (Lorenzen 1967; Owen and Falkowski 1982). Pheophytin forms when the

central magnesium atom in the chloropigment is replaced with hydrogen (Gross 1991). Gross (1991) discussed in a review of chlorophyll synthesis that the process includes eleven possible steps namely: 1) formation of δ- aminolevulinic acid; 2) pyrolle (porphobilinogen) formation; 3) cyclic tetrapyrrole formation (uroporphyringen III); 4) porphyrin side-chain modifications; 5) oxidation of protoporphyrinogen IX to protoporphyrin IX, 6) magnesium chelation of protporphyrin IX to Mg protophyrin IX; 7) esterification of Mg protoporphyrin IX; 8) -iIsocyclic ring formation (protochlorophyllide); 9) protochlorophyllide reduction to chlorophyllide; 10) esterification of chlorophyllide a and 11) biosynthesis of chlorophyll b.

The catabolism of chlorophylls in higher plants has been widely studied but uncertainty still exists about different enzymes involved and the order of the reactions and products formed. The enzyme chlorophyllase has been found in all green vegetables (Mayer 1930), and catalyzes the hydrolysis of phytol esters of chlorophyll and pyrochlorophylls, pheophytins and pyropheophytins. Fang *et al.* (1998), however, studied the chlorophyllase activities and chlorophyll degradation during leaf senescence and came to the conclusion that chlorophyllase activity does not directly regulate chlorophyll degradation (Fiedor 1992; Rodriguez-Amaya, 1987). Lorenzen (1967) showed the occurrence of another enzyme, magenesium-dechelatase, which catalyzes the removal of magnesium from chloropigments. Eckhardt *et al.* 2004 suggested the first step of chlorophyll breakdown to be the removal of the hydrophobic phytol chain catalysed by chlorophyllase to form chlorophyllide. The second step the release of the central Mg atom which is catalysed by Mg-dechelatase, to form pheide. Hötensteiner (2006) mentioned in his review on chlorophyll degradation that the breakdown of chlorophylls qualifies as a detoxification mechanism during senescence, which is vitally important for plant development and survival. Furthermore he described chlorophyll degradation as consisting of four common steps. These steps entail the formation of a primary fluorescent tetrapyrrole intermediate, followed by mostly specie-specific modification of tetrapyrrole side chains. Finally, fluorescent catabolites are excreted into the vacuole, where they non-enzymatically tautomerize to the final non-fluorescent catabolites.

The main obstacle to research in understanding the steps of chlorophyll degradation is that it occurs very rapidly and yields as end products colourless, low molecular-weight compounds such as CO_2, NH_3 and H_2O. The overlapping of the degradation products of chlorophyll with degradation products of other substances make it even more difficult (Gross 1991). Chlorophyll a degrades more rapidly than chlorophyll b (Gross 1991). Consequently, the ratio of chlorophyll a to b is continuously shifted to lower values during

leaf senescence. Moreover, breakdown and synthesis of thylakoid membranes and their lipids in leaves occurs during the natural daylight growth of plants. The turnover however, is not visible, because the decomposition at night is compensated for by new synthesis during the day. The biological half-time of chlorophyll has been calculated to have values from 2.5 days to 7 days (Lichtenthaler and Grumbach 1974).

2.4 GRAPE BERRY DEVELOPMENT AND MATURATION

2.4.1 RIPENING CYCLE AND BEHAVIOUR OF IMPORTANT COMPOUNDS

During ripening, grape berries display different modifications in size, colour, composition, flavour and texture. Berries follow a double sigmoid growth curve (Coombe 1992). Firstly, cell division and later cell expansion is responsible for berry growth. The first rapid growth phase takes place from flowering and reaches its maximum approximately 60 days afterwards. During this rapid growth phase the berry is formed, the seed embryos are produced and several solutes accumulate especially tartaric and malic acids (Possner and Kliewer 1985). Tannins (Kennedy *et al.* 2000a; 2000b; 2001) and other compounds such as minerals, amino acids, micronutrients and aroma compounds also accumulate during the first growth phase (Conde *et al.* 2007). The first growth phase in most cultivars is followed by a lag phase, the duration of which is cultivar-specific and ends in correspondence to the end of the herbaceous phase of the fruit. A second growth phase follows after the lag phase when the most dramatic changes in the berry composition take place, which coincides with veraison or onset of ripening.

Berries almost double in size from veraison to harvest, and become softer, less acidic and in the case of red varieties start to show colour. The solutes that accumulated during the first growth phase can remain until harvest or can be diluted by the great increase in berry volume during the second growth phase. However, some compounds produced during the first growth phase reduce in quantity (on a per berry basis) which is not a result of dilution. A good example of this is malic acid, which is metabolized as an energy source during the second growth phase (Hawker 1969) and is significantly reduced in comparison to tartaric acid, the content of which stays almost constant after veraison (de Bolt *et al*. 2006)

Tannins in the hypodermal tissue seem to be synthesised very early in berry development and change very little from veraison to harvest on a per berry basis (Habertson *et al*. 2002). The evolution of tannins in three Italian cultivars (sum of (+) catechin and (-) epicatechin analysed by HPLC) were initially low (1mg/100g dry weight (dw)), a peak corresponding to veraison was observed, then a rapid decline occurred to

final concentrations between 10 to 20 mg/100g dw (Giovanelli and Brenna 2007). Recent research on the comparison of different analytical methods in measuring condensed tannins in grape skin shows great variability between 36 cultivars and the 3 different methods of measuring skin tannins (Sedon and Downey 2008). Sedon and Downey 2008 conclude that each method potentially analyses a different fraction of the total extractable tannins in grape skin. These results can possibly explain the controversy regarding tannin measurement in the literature. According to Conde *et al.* (2007) the most important event occurring in the second growth phase is the major increase in hexose sugars, such as glucose and fructose, which indicate a total biochemical shift in metabolism to fruit ripening and senescence.

Chlorophyll and carotenoid content of grape berries decreases with ripening especially from veraison to harvest (Bindon 2004; Bureau *et al*. 1998; 2000; Oliveira *et al*. 2004; Razungles *et al.* 1998) and potentially forms C_{13}-norisoprenoids (Baumes *et al.* 2002) and abscisic acid (Marin *et al.* 1996).

2.4.2 RIPENING HORMONES

Endogenous hormones are more abundant than others at specific stages of fruit development and ripening, and play a role during the developmental stages of grape berries. The developmental hormones auxin, cytokinin and gibberellins promote cell division and cell expansion. These hormones are mostly produced by the seeds although there is a possibility that they can be imported into the berry via loading to xylem (pre-veraison) and phloem from the vegetative organs (Conde *et al.* 2007). Just before veraison these hormones reach their peak from which point they decrease sharply through the rest of ripening (Coombe 1992; Blouin and Guimberteau 2000; Wheeler *et al*. 2009). Conversely Coombe and Hale (1973) reported a considerable accumulation of ABA after veraison which plays a role in seed maturation, acquisition of seed dormancy, and possibly resistance to water stress deficit at later stages of ripening as well as the control of maturation (Coombe and Hale 1973).

There are three hormones which can be associated with the regulation of grape berry maturation processes namely: abscisic acid (ABA), ethylene (Szyjewicz *et al.* 1984) and brassinosteroids (Synoms *et al.* 2006). There is a close relationship between the metabolic pathways (Figure 2.4) as well as the chemical structure of carotenoids and the plant hormone, abscisic acid (ABA), which regulates stress responses in plants (Armstrong and Hearst 1996). Furthermore Antolin (2003) found that ABA increases in grape berries under water stress. Little is known about the regulation of carotenoid compartmentalisation and

metabolism towards ABA production under stress conditions. Hypothetically, under conditions where ABA is actively synthesised in plant tissue, carotenoid pools may be increased.

Lund *et al.* (2008) demonstrated via real-time RT-PCR analyses that up-regulation of a 9-*cis*-epoxycarotenoid gene family member, VvNCED2, in grape seed and pericarp and a putative ortholog to a reported abscisic acid receptor, VvGCR2, are correlated with ripening initiation. In higher plants, ABA is derived from C_{40}-*cis*-epoxycarotenoids, either 9'-*cis*-neoxanthin or 9-*cis*-violaxanthin or both, which are cleaved by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin, the direct C_{15} precursor of ABA (Cutler and Krochko, 1999; Liotenberg 1999; Taylor *et al.* 2000). The abscisic acid-deficient mutant aba2 of *Nicotiana plumbaginifolia* is blocked in the epoxidation reaction of zeaxanthin to violaxanthin, indicating that carotenoid precursors are essential for abscisic acid biosynthesis (Marin *et al.* 1996). However, this has not yet been studied in relation to carotenoid and C_{13}-norisoprenoid metabolism in grapes. A more recent study on the relationship between expression of abscisic acid biosynthesis genes, and berry ripening reported that berries may have the potential to synthesise ABA *in situ*. However, the expression profile of the genes (VvCED1, VvNCED2, VvZEP) studied did not correlate well with ABA levels indicating that ABA accumulation is under more complex control (Wheeler *et al.* 2009). Furthermore ABA appears to influence the expression of genes in the anthocyanin pathway and the transcription of genes and activity of proteins involved in sugar accumulation and metabolism during ripening are also influenced by ABA (Cakir *et al*. 2003; Pan *et al*. 2005; Yu *et al.* 2006)

Coombe (1989) found that endogenous ABA concentration rises coincidentally with sugar increase and berry softening and when berries were treated with ABA the onset of ripening was hastened. These results are good evidence in favour of ABA as a hormonal trigger of ripening in grapes.

2.4.3 BIOSYNTHESIS AND DEGRADATION OF CAROTENOIDS IN GRAPE BERRIES

Several oxidative cleavage reactions of carotenoids are known in higher plants. Cleavage of epoxy-carotenoids such as violaxanthin and neoxanthin initiates the synthesis of abscisic acid (ABA). The ABA-deficient mutant aba2 of *Nicotiana plumbaginifolia* is blocked in the epoxidation reaction of zeaxanthin to violaxanthin indicating that carotenoid precursors are essential for ABA biosynthesis (Nussaume *et al.* 1996). In grape berries there is a close relationship between the rate of carotenoid degradation and the generation of C_{13}-norisoprenoids with the onset of grape maturity (Figure 2.6) (Baumes *et al.* 2002).

Furthermore Baumes *et al.* (2002) suggest that carotenoids are the precursors to C_{13}-norisoprenoid glycolates (C_{13}-norisoprenoid bound to glucose) (Figure 2.6). Baumes *et al.* (2002) also studied the biogeneration of C_{13}-norisoprenoids from carotenoids as precursors by means of ^{13}C-labelling and isotopic ratios. It has been found that the configuration of asymmetric centres and axes are common to C_{13}-norisoprenoids and their corresponding carotenoids. ^{13}C markers transferred from carotenoids to norisoprenoids in berries between veraison and maturity also support this model (Baumes *et al.* 2002). Carotenoids are synthesised in grape berries from set until veraison from which point onward they start to degrade to maturity to produce glycosylated C_{13}-norisoprenoids and other intermediate degradation products (Baumes *et al.* 2002).

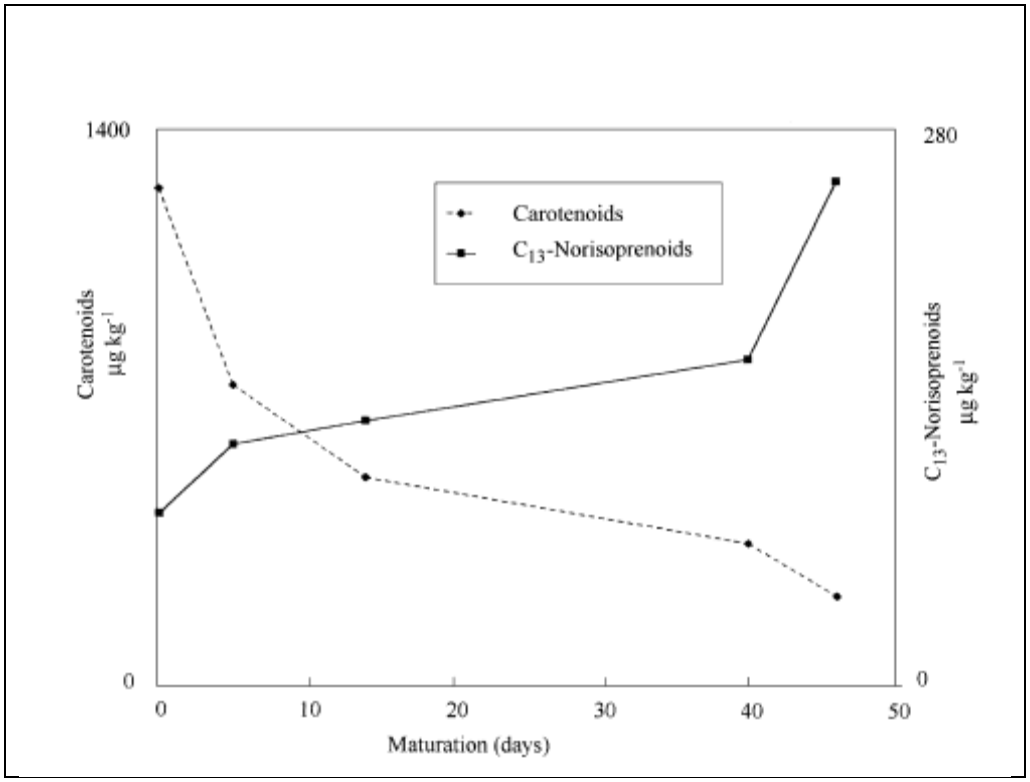

Figure 2.6 Change in levels of carotenoids and C_{13}-norisoprenoid glycoconjugates during the maturation of Muscat berries (Baumes *et al*. 2002).

The biogenetic pathway proposed by Baumes *et al.* (2002) for the degradation of carotenoids to C_{13}-norisoprenoids has three steps. Firstly, the enzymatic degradation of carotenoids by oxidases with the primary product being C_{13}-norisoprenoids carbonyls possessing the oxidised backbone of their carotenoid precursor. Secondly, their modification by oxidases and reductases, depending on the degree of oxidation of the primary product (C_{13}-norisoprenoids carbonyls) and lastly, the glycosylation by glycosyltransferases of those norisoprenoids which contains a hydroxyl group. However,

no such systems are yet described for grapes. A more recent discovery indicated the potential generation of β-ionone from zeaxanthin following cleavage of the latter by a characterized cv. Shiraz carotenoid cleavage dioxygenase (Mathieu *et al.* 2005). Marais *et al.* (1992) showed that the carotenoid lutein might be an original precursor of TDN in his study of the breakdown of lutein in a heated model wine solution. Oliveira *et al.* (2006) shown in his work on eight cultivars from the Douro Valley in Portugal that cultivars with low carotenoid content correspond to wines with higher levels of the grape-derived C_{13}-norisoprenoid volatiles β-ionone, TDN and vitispirane. In grape berries the xanthophyll cycle was initially thought to be active only after veraison since violaxanthin could not be detected before veraison (Razungles *et al.* 1996). However later research where sun-exposed and shaded grapes were studied before and after veraison, it has been found that the carotenoid pool size adjusts sensitively to ambient conditions before veraison. The xanthophyll cycle potentially loses this sensitivity to ambient conditions and therefore potentially its importance with the onset of ripening (Düring and Davtyan 2002).

2.4.4 BIOSYNTHESIS AND DEGRADATION OF CHLOROPHYLLS IN GRAPE BERRIES

In grape berries, Downey *et al.* (2004) found that chlorophyll starts to decrease per berry from two weeks after veraison until the fourth week post veraison to approximately 50% of the original concentration and remains at this level until harvest. Degradation products of chlorophyll, pheophytin a and pheophytin b in grapes are reported by Mendes-Pinto *et al.* (2005). It is unsure if these chlorophyll breakdown products of chlorophyll do exist in grape berries or if they are artefacts of berry sample processing. Giovanelli and Brenna (2007) found in their study on three Italian grape varieties that chlorophyll decreased in all cultivars and almost disappeared in mature white grapes. However, in red grapes a level of 14 to 20% of the initial concentration was found at maturation.

In Shiraz grape berries, Downey *et al*. (2004) found less chlorophyll in berries excluded from sunlight throughout the ripening season compared to berries exposed to sunlight showing that chlorophyll synthesis in grape berries is light-induced (Zucker 1972; Raven 1992). Plant species exposed to sun tend to have a higher chlorophyll a/b ratio (3.2 to 4) compared to shaded plants (2.6 to 3.2) (Lichtenthaler 1971, Lichtenthaler *et al.* 1981). The increased proportion of chlorophyll b in shade plants is due to its absorption properties. Since chlorophyll b absorbs strongly in the 450-480 nm range, it can captures light at low intensity effectively, partially filling the gap in the chlorophyll a spectrum.

2.5 VITICULTURAL INFLUENCES ON CAROTENOIDS AND CHLOROPHYLL CONTENT OF GRAPE BERRIES

2.5.1 THE EFFECT OF SUNLIGHT AND TEMPERATURE

2.5.1.1 The effect of sunlight and temperature on carotenoids and its C_{13}-norisoprenoid degradation products

A significant amount of research has been done on the effect of sunlight on grape berry composition through maturation. Sunlight enhances carotenoid degradation (Razungles *et al.* 1998; Bureau *et al.* 1998; Bindon 2004; Bureau *et al*. 2002; Oliveira *et al*. 2004). Light utilization and thermal dissipation of field-grown sun- and shade-adapted/exposed berries of cvs. Kerner (white) and Porugieser (red) were studied by Düring and Davtyan (2002). This study showed significant divergence of the pool size of the xanthophyll cycle pigments during the development of sun- and shade-adapted berries pre-veraison. Under clear, warm-weather conditions in shade-adapted/exposed berries the xanthophyll pool size decreased to low levels, while in sun-adapted/exposed berries it increased to maximum values shortly before (cv. Kerner) or at veraison (cv. Portugieser) and subsequently declined. The xanthophyll pool size decreased for both cultivars during a rain period suggesting that the xanthophyll pool size varies according to ambient conditions. It was concluded that unripe, sun-exposed berries are better adapted to higher light intensities than shade adapted berries due to their higher capacity for photosynthetic energy consumption and thermal energy dissipation. At the onset of ripening these photo-protective mechanisms appear to lose importance (Düring and Davtyan 2002).

In a study by Steel and Keller (2000) grape berries of cv. Cabernet Sauvignon covered by a UV-B screen which reduced UV light by 98% showed a more pronounced degradation of β-carotene from veraison onward, compared to the same berries under normal light conditions. In this study, lutein also decreased when fruit development occurred under the UV-B screen compared to normal light conditions. Tevini and Teramura (1989) reported in their work, that it is generally accepted that increasing UV-B levels will lead to enhanced overall carotenoid levels in plants, but it is possible that the relative amounts of individual carotenoids can be altered.

Considering the strong relationship between carotenoid degradation and C_{13}-norisoprenoid production, it is conceivable that where environmental factors have an impact on the carotenoid metabolism, C_{13}-norisoprenoid formation would be influenced in likewise manner. Increased light at the bunch zone has been correlated with the increase in the C_{13}-norisoprenoid content of berries and the corresponding wines in some studies

(Baumes *et al.* 2002; Bureau *et al.* 1998; Razungles *et al.* 1998; Marais 1992b; Bureau *et al.* 2000; Ristic *et al.* 2007).

The content of hydrolytically-released C_{13}-norisoprenoids measured in totally shaded (bunches covered with boxes from set to harvest) fruit were decreased in comparison to sun-exposed fruit (Bindon 2004). The decrease of C_{13}-norisoprenoids compared to the sun-exposed fruit correlated with a decreased content of β-carotene and lutein in the berries, showing that shade inhibited carotenoid accumulation, and therefore possibly the pool available for degradation to C_{13}-norisoprenoids. However, since light is known to accelerate carotenoid breakdown after veraison, this result most likely reflects reduced carotenoid synthesis (Bindon 2004).

A study by Bureau *et al.* (2000) on cv. Muscat compared artificially shaded bunches covered with shade cloths to berries under naturally shaded or sun-exposed ambient conditions. The artificially shaded fruit showed a decrease in free and glycolysated C_{13}-norisoprenoids when compared to naturally shaded and sun-exposed berries, which had similar levels of C_{13}-norisoprenoids. Another study which looked at whole-vine shading showed changes in the relative composition of bound C_{13}-norisoprenoids as a proportion of the total C_{13}–norisoprenoids, without affecting the total concentration of C_{13}-norisoprenoids (Bureau *et al.* 2002). However, in the same study, when sun-exposed bunches during set to veraison were compared to the treatment where only bunches were directly shaded, an increase was seen in the total concentration of C_{13}–norisoprenoids in sun-exposed matured berries. The increase of C_{13}–norisoprenoids was 16-36% for sun exposed fruit compared to fruit grown under conditions of extreme shade (10% sun) (Bureau *et al.* 2002). This shows that the clear effect of sunlight on carotenoid catabolism and C_{13}-norisoprenoid production was only evident when comparing extreme conditions of sunlight and of shade. In general, the literature shows variable results in terms of C_{13}-norisoprenoid generation and sun-exposure when intermediate levels of shade and sun-exposure are compared, and a clear relationship between the two factors under ambient conditions has not been observed to date. However Marais *et al.* (1992a) studied the effect of sun-exposed and natural shaded grape bunches on the C_{13}-norisoprenoid content of cvs. Chenin blanc and Weisser Riesling. Marais conclude that with a few exceptions, norisoprenoids concentrations were significantly higher in sun-exposed grapes than in the shaded grapes.

Ristic *et al.* (2007) studied the effect of extreme, artificial shading on anthocyanin, tannin and some C_{13}-norisoprenoids in cv. Shiraz berries and the corresponding wines. Bunches were enclosed with boxes just after flowering, and little effect on the timing of

berry ripening and accumulation of sugar was found. However at harvest the shaded bunches had smaller berries and higher seed weight, juice pH and titratable acidity. When sunlight was excluded from Shiraz berries the amount of anthocynin was not significantly altered, although the composition was shifted towards dioxygeneated anthocyanins (glycosides of cyanidin and peonidin derivatives). However a decrease in skin tannins and an increase of seed tannins were observed. The wines made from the shaded grapes had decreased levels of glycosylated β-damascanone and TDN (C_{13}-norisoprenoids), less anthocyanins and tannins, and altered sensory attributes. A similar study by Downey *et al*. (2004) on cv. Shiraz berries showed the same results for anthocyanin but no significant difference in seed and skin tannin was observed.

From these experiments it seems clear that sunlight may influence the formation and degradation of carotenoids and C_{13}–norisoprenoids, mainly when extremes in the levels of sun-exposure are evaluated.

2.5.1.2 The effect of sunlight and temperature on chlorophyll content of grape berries

Chlorophyll content of berries influenced by sunlight and temperature has received less attention in grapes than has carotenoids with only a few studies to date. Downey *et al.* (2004) studied the effect of shading on berry development and flavonoid accumulation in Shiraz berries by enclosing bunches one week after flowering. It was found that the concentration of chlorophyll when expressed as mg/g fresh weight of berry was higher earlier in berry development and then decreased as the berry ripened in both shaded and sun-exposed fruit. Total chlorophyll per berry increased from flowering until one week before veraison for the exposed fruit, which coincided with the first phase of berry growth. Until two weeks post-veraison the chlorophyll content per berry remained relatively constant, where after chlorophyll decreased approximately 50% to the fourth week post-veraison, and remained at this level until harvest. The chlorophyll concentration in shaded fruit was substantially lower than in the sun-exposed fruit throughout berry development. Moreover, in shaded fruit there was only a slight increase in chlorophyll during the period corresponding to chlorophyll accumulation in the exposed fruit and a decrease post-veraison to almost zero. Less chlorophyll would be expected in shaded fruit since chlorophyll synthesis is light-induced (Zucker 1972, Raven 1992).

2.5.2 THE EFFECT OF VIGOUR, PLANT WATER STATUS AND SOIL TYPE

Winkler (1974) gave the following definition for grapevine vigour as:

"the quality or condition that is expressed in rapid growth of a part of the vine. It refers essentially to the rate of growth.…the vigour of shoots of a grapevine varies inversely with the number of shoot and with the amount of crop.…the quantity of action with respect to the total growth and total crop of which the vine or a part of it is capable."

Dry and Loveys (1998) stated that low vigour on a single shoot basis can be considered when a shoot is thin and short and has few and small leaves. On the other hand when shoots tend to have rapid shoot growth in spring which may be prolonged well in to the growing season, often extending post-veraison they can be considered as having high vigour. Huglin (1986) recommended pruning mass as a measure of vigour.

Excess vigour can be problematic especially in mature vines trained on a restrictive trellis system since these vines have dense canopies which result in high within-canopy shading (Dry and Loveys 1998). These conditions can have a detrimental effect on fruit quality and composition, and in turn affect crop load (Dry and Loveys 1998). Furthermore, reduced fruit initiation in the buds can occur (May 1965) as well as development of early bunch stem necrosis (Jackson 1991). In berries, high vigour can result in reduced sugar and tartrate concentrations, with higher malate and increased potassium concentration which leads to higher pH, and potentially lowered phenolic and flavour compounds in wine (Rojas-Lara and Morrison 1989; Dokoozlian and Kliewer 1995). Undesirable 'vegetative' character in wines made from Cabernet Sauvignon and Sauvignon blanc grapes can be the due to unbalanced high vigour vines (Allen *et al.* 1996).

The amount of sunlight infiltrating the canopy of a grapevine is closely related to vigour, since high vigour vines which are not well accommodated by their trellis system will have denser canopies. This is in particular reflected in the amount of light which infiltrates to the bunch zone, which is less in high vigour vines when compared to less vigorous vines. Irrigation can induce excessive vigour if irrigation is not well scheduled and managed according to soil moisture. However, Dry and Loveys (1998) state that excessive vigour cannot be successfully managed by deficit irrigation strategies alone. The application of deficit irrigation seems to significantly reduce yield and the minor improvement in fruit quality which may be reflected in increased berry anthocyanins may not be sufficient to increase, or even maintain, economic return. Goodwin and Jerie (1992) studied regulated deficit irrigation and stated that the main yield component affected was berry weight and in the cases of no significant reduction there was little or no effect in vegetative growth (vigour). Post-veraison water deficit has little or no effect on shoot growth (Matthews and Anderson 1989; Poni 1994; Noar 1993) because the canopy development is largely complete by veraison (Sommer and Clingeleffer 1996). The correct

combination of soil, rootstock and cultivar can be an important aspect to prevent excessive vigour on certain terroirs with fertile soil.

Little research has been done on the effect of vigour on carotenoids and chlorophyll content of berries per se, although inferences can be made as to the potential effects of vigour on their metabolism based on vine microclimatic effects. Oliveira *et al*. (2004) found that grapes grown with higher vegetative height appear to have higher carotenoid levels while grapes from grapevines with lower vegetative height had berries which were heavier and contained more sugar. It is explained by Oliveira *et al*. (2004) that higher vegetative height canopies are denser and allow less sunlight into the bunch zone than lower vegetative height canopies. However, higher sugar levels may also reflect a later developmental stage, or accelerated ripening, which would in turn reflect lower carotenoid levels, so the results cannot be interpreted conclusively.

As discussed previously, a component of vigour is reflected in the plant water status of the grapevine. Most studies on grapevine water status compare deficit irrigation strategies or non-irrigated vines with irrigated grapevines. Post-veraison water deficit has little or no effect on shoot growth (Matthews and Anderson 1989; Poni 1994; Noar 1993) because the canopy development is largely complete by veraison (Sommer and Clingeleffer 1996). Excessive stress imposed after veraison may lead to reduced sugar accumulation and increased pH (Williams and Matthews 1990) as well as a decrease in yield (Naor 1993). Furthermore Koundouras *et al*. (2006) showed that differences in vine water status (measured through predawn leaf water potential) were highly correlated with the earliness of shoot growth cessation and veraison.

Through historical research, it is widely acknowledged that smaller berry size can be achieved through deficit irrigation which can play a role in wine quality, based on the concept that surface area/volume ratio of the berries decreases with the increase of berry size (Ojeda *et al.* 2002). A restriction in cell wall development, and thus the growth of berries post-veraison is inhibited when subjected to water deficit resulting in smaller berries. Many important compounds which contribute to wine quality are situated in the grape skin, tannins, athocyanins, carotenoids, chlorophylls and many aroma precursors, such that smaller berries induced by water deficit have a potentially greater relative solute to solvent ratio than larger berries (Ojeda *et al.* 2002). However Roby (2004) came to the conclusion that the effect of vine water status on the concentration of skin tannins and anthocyanins is greater than the effect of fruit size per se. Skin and inner mesocarp tissue respond to water deficit in terms of their differential growth, although there may be a direct stimulation of phenolic biosynthesis (Roby, 2004). Thus, the response of grape berry

secondary metabolites to water deficit can be two fold: an indirect and positive response due to the effect of berry size (a concentration effect) and a direct response on the biosynthesis that can be either positive or negative, depending on the type of secondary compound, degree of water deficit, and the period during which it is applied (Ojeda *et al.* 2002).

Sugar accumulation and the onset of anthocyanin synthesis can be accelerated by early water deficit (before veraison). Gene expression profiling showed that an increase in anthocyanin accumulation results from earlier and greater expression of some genes in response to water deficit, those which control flux through the anthocyanin biosynthetic pathway (Castellarin *et al.* 2007). When excessive water deficit occur post-veraison, fruit sugar is often reduced (Noar *et al.* 1993). Koundouras *et al*. (2006) also found in their study on a Greek cv. Agiorgitiko grapevine cultivar that early water deficit during the growth period has beneficial effects on the concentration of anthocyanins and total phenolics in berry skins. Koundouras *et al*. (2006) found that water deficit accelerates sugar accumulation and malic breakdown in juice. Sugar unloading in berries is inhibited in ripening berries during water deficiency stress (Wang *et al.* 2003).

Oliveira *et al.* (2003) studied the effect of water deficit on carotenoids in the grapevine cv. Touriga Nacional. This experiment compared the carotenoid composition of fruit from non-irrigated versus irrigated vines on a high water-retention capacity soil and a low water retention capacity soil. The deficit treatment caused a reduction in fruit weight that was independent of soil type. Oliveira *et al.* (2003) stated that the reduction in berry weight can be due to less sugar in the berries or due to restriction in cell expansion. Oliveira *et al.* (2003) found that berry carotenoid content was increased up to 60% by the non-irrigated treatment when the soil had a low water-retention capacity. On the high water-retention capacity soil, there was no effect on carotenoid content comparing irrigated and non-irrigated treatments, albeit an observed reduction in berry size. Water stress caused an increase in carotenoid content for all the carotenoids analysed: lutein, β-carotene, neoxanthin, violaxanthin and luteoxanthin (Oliveira *et al.* 2003). Oliveira *et al.* (2003) showed that the response of the carotenoids to water stress occurred in fruit from an early stage of development, and the effect on carotenoid content was retained as the fruit matured.

PRD-irrigation (partial root zone drying) influences on carotenoids in Cabernet Sauvignon were studied by Bindon *et al.* (2007). Fruit weight decreased 10-20% in response to PRD treatment for both seasons studied, and was found to be associated with small increases in the concentration of the carotenoids β-carotene and lutein either at

certain stages during grape ripening or at harvest. An increase in concentrations of TDN and β-damascenone, both C_{13}-norisoprenoid degradation products of carotenoids, were observed over two seasons of the study. Carotenoids and C_{13}-norisoprenoids are concentrated in the berry skin (Razungles *et al.* 1988) that a change in skin to fruit ratio could increase the relative concentration of these compounds per gram in smaller fruit. Bindon *et al.* (2007) concluded that biochemical changes as result of PRD caused an increase in the C_{13}-norisoprenoids concentration and were most likely indirectly related to increased biosynthesis of carotenoid precursors, and not just to a change in berry weight alone (Bindon *et al.* 2007). Bindon *et al.* (2007) showed that a deficit irrigation treatment (PRD) could result in an increase in both carotenoids and C_{13}-norisoprenoids in Cabernet Sauvignon berries. In the current research record, there is no report on the effect of grapevine water deficit on chlorophyll content of grape berries.

2.5.3 THE EFFECT OF TERROIR AND CULTIVAR SELECTION

"Terroir can be defined as a spatial and temporal entity, which is characterized by homogeneous or dominant features that are of significance for grape and/or wine; i.e. soil, landscape and climate, at a given scale-duration, within a territory that has been found…..and genotype related technical choices" (Vaudour 2001).

The effect of altitude (terroir) and different cultivars (Tinta Amarela, Tinta Barroca, Souzão, Touriga Franca, Touriga Nacional, Tinta Roriz, Tinto Cão, Touriga Fêmea) on the carotenoid content of grape berries in the Douro Valley Portugal has been studied by Oliveira *et al.* (2004). Oliveira *et al.* (2004) I found that high-elevation terraces, which present lower temperature and higher humidity during the maturation period, produce grapes with higher carotenoid content. The cultivars Touriga Brasileira and Tinta Amarela produced higher concentrations of carotenoids for the two seasons studied, although other cultivars (Tinta Barroca, Souzão, Touriga Franca, Touriga Nacional, Tinta Roriz, Tinto Ca˜o) were not consistent in their response to elevation relative to one other. The variance between cultivars may have been due to differences in climate for the two seasons, or the combined effect of climate (sun-exposure, temperature, wind and rainfall), soil type and vigour. In a later study by Oliveira *et al.* (2006), the same cultivars were studied for three consecutive vintages and showed differences in berry carotenoid content between cultivars and for the same cultivar between vintages. Cultivars Touriga Fêmea, Tinta Amarela and Tinta Barocca were found to have higher carotenoid content than cvs. Touriga Nacional, Souzão and Tinto Cão.

Limited research has been done on the effect of terroir and cultivar on carotenoid content in grape berries therefore the impact on the C_{13}-norisoprenoids will be discussed.

The influence of the climate and the soil (terroir) on C_{13}-norisoprenoids profile of two white varieties Alvarinho and Loureiro, in two sub-regions were studied by Araujo (2004). Araujo (2004) calculated the different climate indexes for each year of study from which some of the plots fell into different categories for the different seasons. By this, he showed how dramatically climate can differ from season to season. The individual C_{13}-norisoprenoids differed greatly between seasons. Principal component analysis showed that global temperature had the strongest influence on the volatile compounds of grapes and soil to a lesser extent (Araujo 2004). It is most likely if the individual C_{13}-norisoprenoids differed greatly between seasons that their precursors (carotenoid) synthesis or breakdown were also greatly affected, although this is speculative.

The effect of different terroirs in the Rhone Valley on the volatile compounds of cv. Grenache wines were studied by Sabon *et al.* (2002). The findings suggested two major groups of wine: firstly the wines from the southern zone with warmer climate where maturation occurs early which contains the highest amount of β-damascenone and sugar but low total acidity. The other group consist of wines from soils producing grapes that mature later, and wines with higher amounts of β-ionone and total acidity and lower levels of sugar and β-damascenone Differences in the C_{13}-norisoprenoids β-damascenone and β-ionone for different cultivars, terroirs and vintages in wine were observed by Kotseridis *et al.* (1999a, 1999b). It is suggested that the influence of climate or global temperature of the specific vintage may be responsible for this variance. Oliveira *et al*. (2006) studied the C_{13}-norisoprennoids TCH, β-damascenone, TDN, vitispirane and β-ionone, and related carotenoid content of berries of eight representative Portugese grape varieties (Tinta Amarela, Tinta Barroca, Souzão, Touriga Franca, Touriga Nacional, Tinta Roriz, Tinto Cão, Touriga Fêmea) of the Douro Region in Portugal for three consecutive years. Different amounts of C_{13}-norisoprenoids for the same cultivar between vintages were observed as well as between cultivars. Specific cultivars showed higher amounts of some of the C_{13}-norisoprenoids than others, for example Touriga Nacional, Sousao and Tinta Cao appear to have higher content of free norisoprenoids, namely β-ionone for Touriga Nacional and conversely vitispirane and TDN predominate in Sousão and Tinto Cão. Since the C_{13}-norisoprenoid derivatives may reflect a variation in the content of their parent carotenoid precursor in grapes, this shows that there may be a potential influence of grape carotenoid profile on cultivar-specific wine aroma as it relates to isoprenoids.

Marais *et al*. (1992) studied the effect of different cultivars, vintages and regions on the TDN content of wines. It was found that cv. Chenin blanc and Cape Riesling contained relatively low TDN concentrations compared to Kerner. Weisser Riesling from Italy

contained lower concentrations of TDN compared to Weisser Riesling from Germany. While South Africa, a warm climate country, had an average of 78% higher TDN concentrations in the wine compared to the cool climate European countries. These results suggested that there is a difference in the rate of development in TDN precursors of these countries due to the combined effect of climatic conditions and viticultural practices.

Temperature has an influence on the C_{13}-norisoprenoid content of grapes, but this is difficult to separate from light since sun-exposed berry temperatures will potentially differ from those in the shade. Smart and Sinclaire (1976) found that berries exposed to direct sunlight during the day can be up to 15 ºC warmer than the ambient temperature, and decrease up to three degrees lower than ambient temperature at night. However, Coombe and Iland (1987) came to the conclusion that temperature is the most important environmental factor influencing grapevine cultivation. The effect of climate has the greatest effect on grape composition followed by soil and cultivar (Van Leeuwen 2004). The effect of climate and soil on vine development and grape composition can largely be explained by their influence on vine water status through rainfall (climate) and water holding capacity (soil). Giovanelli and Brenna (2007) studied the evolution of some phenolic components, carotenoids and chlorophyll during the ripening of three Italian grape varieties (Barbera, Nebbiolo– red and Erbaluce- white) and observed very similar profiles of the studied compounds for all three cultivars. This observation implies that climatic conditions and sun exposure play an important role in the evolution of these compounds.

2.6 RECENT ADVANCES OF ANALYTICAL TOOLS AND TECHNIQUES TO ASSESS AND MEASURE GRAPE RIPENESS

Up to now spectrophotometric and liquid chromatographic methods have been mainly used for plant pigment analyses (Schoefs 2002; Sander *et al.* 2000; Cserhati and Fogacs 2001). HPLC is mostly used to analyse plant pigments due to its high reproducibility and low detection limit (Breithaupt 2004; Belie *et al.* 2003; Taylor *et al.* 2006; Van den Berg *et al.* 2000). So far the best separation of various carotenoids and their isomers (Emenhiser *et al*. 1999) has been attained on a C-30 chemically-bonded phase. For carotenoid RP-HPLC, mixtures of organic solvents are used with methanol and acetonitrile or mixtures thereof as main components (Van den Berg *et al.* 2000). However, HPLC methods require extensive sample preparation, including solvent extraction of the pigments, which are usually strongly bonded to other plant constituents (e.g. proteins). Therefore the analysis

results may not represent the actual carotenoid content. Furthermore, pigments are sensitive to certain solvents, high temperature, light and acidity that may cause the formation of *cis*-isomers of carotenoid and the degradation of chlorophyll to chlorophyll derivatives (Rodriguez-Amaya *et al.* 2008; Van den Berg *et al.* 2000; Oliver and Palou 2000; Gross 1991).

Since grape pigments make an important contribution to wine colour (anthocyanins) (Ribereau-Gayon and Glories 1986) and aroma (carotenoids and chlorophylls as aroma precursors) (Baumes *et al.* 2002; Sefton *et al.* 1993), instant analyses of these pigments can be valuable for rapid determination of optimal ripeness of these pigments, assessment of grape quality and potential wine quality. Techniques like NIR spectroscopy and chlorophyll fluorescence show potential for rapid analysis of carotenoids and chlorophylls... For example research has shown that NIR-FT-Raman (near infrared fourier transform spectroscopy) can give a sensitive detection of the individual carotenoids by Raman Resonance in the visible region when the wave number of the laser excitation coincides with an electronic transition (Withnall *et al.* 2003; Veronelli *et al.* 1995). Raman is a spectroscopic technique used in condensed matter physics and chemistry to study the vibration, rotation, and other low-frequency modes in a system (Gardiner 1989). FT-Raman spectroscopy also gives a strong enhancement of carotenoids due to the known pre-resonance effect; furthermore the disturbing fluorescence effect of biological material usually observed when laser excitation is performed in the visible wavelength range can be avoided (Ozaki *et al*. 1992). Strong bands of carotenoids are observed in the Raman spectrum within the 1500-1550 and 1150-1170 cm^{-1} range due to in-phase C=C and C-C stretching vibrations of the polyene chain (Withnall *et al.* 2003; Veronelli *et al*. 1995). It has been found that FT-Raman spectroscopy can be successfully applied for the identification of carotenoids directly in the plant tissue without any preliminary sample preparation. Furthermore, FT-Raman mapping is able to show the location of carotenoids in the surface layer of the plant tissue and perform semi-quantitative measurements of these carotenoids (Schultz *et al.* 2005).

Furthermore Davey *et al*. (2009) showed in his work on lyophilised banana pulp that it is possible to develop predictive models with visible and near infrared reflectance spectroscopy to determine total carotenoid and β-carotene fractions with r^2 values of 0.84 and 0.89 respectively. However the evaluation of colour measurements with a colorimeter (using visible spectra 380-770nm), FT-NIR and FT-MIR (Ruiz *et al.* 2008) showed better results for developing a prediction model to predict β-carotene in apricots, although low r^2 values with high prediction errors where obtained with FT-NIR and FT-MIR data. In a

further studied Baranska *et al*. (2006) found attenuated total reflection infrared spectroscopy (ATR-IR) recording the range between 650 and 4000 cm^{-1} the most sufficient for predicting lycopene and β-carotene content of tomato homogenate. Accurate prediction (r^2=0.98 and RMSECV of 3.15) of lycopene was obtained by scanning whole tomatoes with visible NIR using the spectra range from 400 to 1500nm^{-1}. It is most likely with the development of new technology and the improvement in these research fields that portable devices for measuring pigments in the field will become more available in the near future. However, for the validation of new, rapid, non-destructive measures, an accurate and reproducible analytical method is required.

A portable device using Raman scattering spectroscopy to determine carotenoid levels as an indication of oxidative deterioration is already patented. This device can give an indication of the general health or stress status in living plants and plant products (Gellerman *et al.* 2004).

Kolb *et al*. (2006) found that chlorophyll florescence measurements are well-suited to determine non-invasively sugar accumulation in white grape berries cv. Bacchus and Silvaner. Studies by Agati *et al*. 2008 included the assessment of anthocyanin in whole grape bunches via chlorophyll fluorescence imaging and showed that a chlorophyll fluorescence imaging method based on pigment screening of excitation is able to determine the distribution of anthocyanins in whole grape bunches. On this basis the assessment of phenolic maturity in the vineyard can be foreseen. This might be a new rapid and non-invasive technique for the assessment of grape ripening and to determine the appropriate time to harvest for optimal colour in grapes. Gitelson and Merzlyak (2002) did non-destructive assessments of chlorophyll, carotenoid and anthocyanin content in higher plant leaves by using reflectance spectroscopy. They established relationships between reflectance and pigment content as well as quantitative techniques for pigment estimation in leaves of different non-related species with a wide range of pigment content and composition. However the applicability of these proposed algorithms to grapes remains to be verified.

Portable colorimeters also showed promise in rapid measurements of chlorophyll in intact leaves since it correlated well with extracted chlorophyll (Yadava 1986; Marquad and Tipton 1987). The use of colorimeters for measuring grape pigments however still needs to be verified. Another technology has been developed by Vivelys society in partnership with Montpellier SupAgro (France), which can assist on profiling berry maturation and determining optimal ripeness. This technology is based on the evolution of the berry tint angle (berry colour evolution), which is determined using optical technologies, as an

indicator of berry ripening versus wine aromatic profile (Deloire *et al*. 2008; Brenon *et al*. 2005). This method is based on an indirect relationship between the evolution of the berry tint angle (according to the HSL model – hue, saturation and luminescence). This technology is currently being used and tested at the commercial level in the Northern and Southern Hemispheres.

Chemometrics is a valuable tool in combination with pigment measurements to explore the relationships of pigments and ripeness parameters in grapes and the viticultural parameters affecting these parameters. Furthermore the potential of pigments to predict other important variables can be evaluated. Many variables can be accommodated in one analysis which presents the data visually making it easier to interpret. Chemometric techniques which can be used to discriminate between samples and explore potential relationships are principle component analyses (PCA) and partial least square (PLS) analysis. PCA and PLS analysis describe sample clustering and detect compounds responsible for the separation of samples (Kemsley 1998). PCA is essentially a descriptive method used to visualise samples present in an n-dimensional space of a set of inter-correlated variables into a smaller number of dimensions, called principle components (PCs), where each principle component (PC) account for a portion of the total variance of the data set (Kemsley 1996; Summer *et al*. 2003). PLS is based on multivariate regression, taking into account the covariance between variables. PLS is regularly used to predict quantitative variables using spectroscopic measurements. PLS regression, like PCA, identifies synthetic variables (scores) that describe the variance in a sample set, but PLS uses additional information: a priori definition of the sample groups. Another output is to reveal the most effective variables that allow the groups to be separated (Kemsley 1998; Roussel *et al.* 2003; Ergon 2004).

Work by Le Moinge *et al.* (2008) is an example of a study that made use of these techniques these authors showed that front face fluorescence spectroscopy and visible spectroscopy coupled with chemometrics had the potential to characterise ripening of Cabernet Franc grapes. Le Moigne *et al.* (2008) stated that visible spectroscopy however appeared to be more appropriate when predicting technological indicators and anthocyanins. These two spectroscopic methods have the advantage to be rapid and could be non-destructive. Moreover, the whole spectrum is analysed and instead of single wavelengths unlike the chlorophyll fluorescence method and thus can detect more ripening changes.

Given that multiple technologies exist, which allow the rapid, non-destructive measurement of grape analytes *in situ*, the possibility exists for calibrations to be

developed for important predictors of grape ripeness, namely carotenoids and chlorophylls, amongst others. However, in order for this research to progress, a robust method for validation of non-destructive measures is needed. A second requirement is for the importance of the target compounds to be evaluated in relation to known viticultural parameters, namely vineyard variability (vigour, soil water) and other significant ripeness measures such as hexose sugars, malic acid and anthocyanins. The current study will outline a research exercise which will evaluate both a HPLC analytical method, as well as the application of this method to an experimental dataset.

2.7 CONCLUSION

The biosynthesis, degradation, structure, location and role of carotenoids and chlorophyll are well studied. Demmig-Adams *et al.* (1996) discussed in his review the time scale in which reactions in the xanthophyll cycle takes place and it varies from a few minutes (de-epoxidation) to hours (epoxidation) in response of various environmental conditions. These sudden fluctuations make it difficult to study individual carotenoids and this factor is important when samples are collected in the field. A record of environmental conditions such as weather conditions, during sample collection needs to be kept.

The effect of viticultural parameters such as soil type, irrigation, vigour and climate on the composition of grapes needs to be studied in more depth - although a lot of research has been done in this field, a lot of uncertainty remains. In this research the main focus will be to explore the changes in the carotenoids profile of grapes during ripening and to attempt to draw correlations with some viticultural factors which could potentially influence carotenoid synthesis and degradation.

A lot of field research has been done on different cultivars and the effect of sunlight on the carotenoid composition of the grape berry and it is clear that sunlight has an enhanced effect on the degradation of carotenoids after veraison to C_{13}-norisoprenoids. Bureau *et al.* (2000) showed that only the comparison of extreme light and shaded conditions had significant differences in carotenoid and C_{13}-norisoprenoid content of cv. Muscat berries. Shaded berries had less initial chlorophyll than berries in direct sunlight and decrease to zero at maturity while chlorophyll was still present in sunlight exposed berries. More research is needed on individual carotenoids and chlorophylls and their response to different light conditions in the canopy as well as the effect of temperature on chlorophyll and carotenoid content.

Different vine vigour levels and canopy densities can imply different intensities of sunlight reaching the bunches which can increase the bunch temperature. No research is currently available on this subject regarding carotenoid and chlorophyll content of grapes. One of the aims of this study is to investigate the difference in berry chlorophyll and carotenoid levels of different vigour level vines of cv. Merlot by monitoring both light infiltration and temperature in the canopy. The effect of water deficit on berry composition is clearly a decrease in berry size and an increase in skin to pulp ratio and thus a concentration effect of compounds situated in the berry skin. Water deficit can also influence the biosynthesis and degradation of some important compounds. More research is necessary to understand the degree of water deficit as well as optimal irrigation times to alter the synthesis and degradation of important compounds in berries. In this study different water deficit levels will be applied to vines to study the response of berry composition. There is a lack of research on carotenoid profiles of specific cultivars on different terroirs. The current study will give an indication of the carotenoid and chlorophyll profile of cv. Merlot and the content per berry of individual carotenoids and chlorophylls through ripening. It has been stated that the annual climate for one region can differ so dramatically that it can fall into two different climate indexes (Araujo 2004).

Current research on simpler, less expensive and reliable methods for analysing carotenoid and chlorophyll content of berries as potential ripening and quality parameters can be valuable. Correlation of carotenoids and chlorophyll content of grape berries with other important ripening parameters will be explored. The possibility of predicting other ripening parameters from carotenoid and chlorophyll content of grape berries will be investigated.

In the current study, the optimisation of an HPLC technique for the combined analysis of grape carotenoids and chlorophylls will be detailed and discussed. This will be followed by application of the analytical method to a limited dataset of grape samples from a single vineyard. The response of the grape carotenoid and chlorophyll profiles to some selected vineyard variables will be explored using chemometric analyses. From this, some general observations relating to the experimental data will be discussed, and the relevance of carotenoid and chlorophyll analysis as valuable ripening predictors will be evaluated. With the availability of new technologies, allowing the rapid, non-destructive measure of grape analytes *in situ*, the potential for further research in this direction will be discussed in the final chapter.

2.8 LITERATURE CITED

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Chapter 3

Technical report

Investigation and optimisation of a method for the extraction and quantification of chlorophylls and carotenoids in grape berries (*Vitis vinifera* cv. Merlot).

INVESTIGATION AND OPTIMISATION OF A METHOD FOR THE EXTRACTION AND QUANTIFICATION OF CHLOROPHYLLS AND CAROTENOIDS IN GRAPE BERRIES (*VITIS VINIFERA* CV. MERLOT).

3.1 INTRODUCTION

Carotenoids and chlorophylls can be widely found in plants, microorganisms, and as by-products of digestion in animals and humans, with more than 700 natural carotenoids known today.

Plants and some micro-organisms can synthesize carotenoids and chlorophylls while animals and humans are incapable of their de novo synthesis but take them in through their diet (Felt *et al.* 2005). Carotenoids have two main functions in the photosynthetic pathway of higher plants: photo-protection and light harvesting. Photo-protection is the channelling of photochemical energy away from chlorophyll whereas light harvesting is the collection and subsequent transfer of light on to chlorophyll via photochemical transduction (Krinsky 1979). These functions are crucial for plant survival since excited triplet molecules can damage the photosynthetic apparatus, and thus requires both the effective transduction of light energy and dissipation of excess photochemical energy. The most common carotenoids present in mature (ripe) grapes are β-carotene and lutein, representing almost 85% of the total carotenoid content (Baumes *et al.* 2002). They are accompanied by minor xanthophylls such as neoxanthin, violaxanthin, lutein-5,6-epoxide, zeaxanthin, neochrome, flavoxanthin and luteoxanthin which make up the remaining proportion of total carotenoids (Baumes *et al.* 2002).

Carotenoids belong to the group of red or yellow pigments which absorb light between 450 – 570 nm in the visible light range (Van den Berg *et al.* 2000). The structure of carotenoids consists of a system of long, aliphatic conjugated double bonds responsible for the biochemical reactivity of these compounds (Van den Berg *et al.* 2000). In natural sources, carotenoids occur mainly in the all-*trans* (all-E) configuration (Chandler and Schwartz 1987). Isomerization of *trans*-carotenoids to *cis*-isomers (all-Z) is promoted by contact with acids, heat treatment and exposure to light (Oliver and Palou 2000; Rodriguez-Amaya *et al.* 2008; Van den Berg *et al.* 2000). These alterations can have profound effects on the configuration and structure of these lipophilic pigments.

The unique role of chlorophyll in photosynthetic light harvesting and energy transduction in higher plants is well known and documented in the literature (Gross 1991). The structure of chlorophyll is a cyclic tetrapyrrole with a structure similar to the heme group of globins (hemoglobin, myglobin) and cytochromes. Chloropigments are susceptible to degradation either chemically or enzymatically. Enzymes, weak acids, oxygen, light and heat can lead to the formation of a large number of degradation products (Gross 1991). Although several types of chlorophyll exist, chlorophyll a is the major pigment in higher plants and chlorophyll b is an accessory pigment. Chlorophyll a and chlorophyll b exist in a ratio of approximately 3:1 in higher plants (Gross 1991).

Extensive research has been done on the carotenoid and chlorophyll content of food products and plants (Taylor and Ramsay 2005). During these studies different analysis techniques, solvents and extraction methods were used (Felt *et al.* 2005; Mendes-Pinto *et al.* 2004; Taylor *et al.* 2006).

The analysis and study of carotenoids in grape berries are important for the wine and grape industry since they were found to be precursors of important (C_{13}-norisoprenoid) aroma compounds (C_{13}-norisoprenoids) present in wine. Furthermore, carotenoids and chlorophylls were found to be potential indicators of berry ripeness (Baume *et al.* 2002; Ferreira *et al.* 2008; Lund *et al.* 2008). Kolb *et al*. (2006) demonstrated that the chlorophyll content of grape berries might be used to predict berry ripeness through chlorophyll fluorescence non-invasive measurements. Thus, it is evident that the analyses of carotenoids and chlorophylls in berries are an important research field for the wine industry.

A method to evaluate both the carotenoid and chlorophyll profiles of lyophilised grape tissue was however not readily available at the outset of the current study.

In this chapter, an existing method for HPLC analysis of carotenoids in *Arabidopsis thaliana* leaf tissue (Taylor *et al.* 2006) together with a combination of the extraction methods used by Oliveira *et al.* (2003) and Mendes-Pinto *et al.* (2005) was optimised for the analysis of carotenoids and chlorophylls in green and red lyophilised berry tissue. Additionally, suggestions for further optimisation and pitfalls of this method will also be discussed.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIAL AND GROWTH CONDITIONS

Plant material used in this study was grape berries sourced from a nine-year-old commercial *Vitis vinifera L. cv.* Merlot vineyard (clone MO 9 clone grafted on Richter 110 rootstock) located in the Stellenbosch region, South Africa. Berries were harvested at different stages of ripening. For later sections, 'green' berry tissue represents berries collected pre-veraison, and 'red' berry tissue represents grapes at harvest (23 to 24 °Brix).

3.2.2 ANALYTICAL MATERIALS

The following solvents were purchased from Sigma-Aldrich (Steinheim, Germany): methyl tertiary butyl ether (MTBE), ethyl acetate, diethyl ether, methanol, hexane, tri-ethylamine and 2, 6-di-*tert*-butyl-4-methylphenol (BHT). All the chemicals used were of HPLC grade with the exception of sodium chloride (Fluka Chemie) and Tris base (Roche Diagnostics, Manheim, Germany) which were of analytical grade. The authentic standards β-apo-caroten-8-al (purity ≥ 96%), zeaxanthin (purity ≥ 96%), β-carotene (purity ≥ 95%), violaxanthin (purity ≥ 90.5%), neoxanthin (purity ≥ 88%), antheraxanthin (purity ≥ 88.4%) and lutein (purity ≥ 94%) were obtained from CaroteNature (Lupsingen, Switzerland). Chlorophyll a (purity ≥ 96%) and chlorophyll b (purity ≥ 94%), were acquired from Sigma-Aldrich (Steinheim, Germany). All the ratios and percentages of solvents are indicated as volume per volume (v/v), unless otherwise stated.

3.2.3 PREPARATION OF STANDARDS

The authentic standards detailed in Table 3.1 were dissolved in their respective solvents with the addition of 0.1% (w/v) BHT. The stock solutions were divided in 1 ml aliquots in to small amber high performance liquid chromatography (HPLC) vials and dried under a stream of nitrogen gas to prevent isomerisation prior to storage at -80ºC (Felt *et al*. 2005). These standards were re-dissolved in the appropriate solvent prior to use. The concentrations of the stock solutions for HPLC analysis are listed in Table 3.1. All dilutions were made in ethyl acetate: methanol (1:4) containing 0.1% (w/v) BHT. All dilutions were kept at -20ºC for no longer than 48 hours and allowed to reach room temperature before analysis.

Table 3.1 The authentic standards used and their solvents.

Authentic standard	Solvent for stock solution	Stock concentration µg/ml
Zeaxanthin	chloroform	100
Violaxanthin	chloroform	100
Antheraxanthin	chloroform	100
Neoxanthin	chloroform	100
Lutein	chloroform	100
β-apo-carotenol-8-al	Ethylacetate:methanol (1:4)	100
β-carotene	Chloroform:hexane (1:9)	100
Chlorophyll a	methanol	50
Chlorophyll b	methanol	50

3.2.4 SAMPLE PREPARATION

Merlot berries were sampled at different stages of ripening (pre-veraison, veraison, post veraison and harvest) from the experimental vineyard (described in detail in chapter 4, section 4.2.8). Berries were immediately frozen after collection in liquid nitrogen to prevent any enzymatic or photo- degradation. While berries were still frozen, their seeds were removed. The berry pericarps were ground under liquid nitrogen to a fine powder with an IKA A11 basic grinder (IKA®-Werke GMBH & CO.KG, Staufen, Germany), where after tissue was lyophilised and kept at -80ºC prior to extraction and reverse phase (RP)-HPLC analysis. Sample preparation was done under subdued light at all times.

3.2.5 EXTRACTION

Extraction for RP-HPLC analysis was done on 100 mg red and 50 mg green tissue to which 500 µl millipore water and 10 µl internal standard (β-apo-caroten-8-al 200 ng/µl) was added prior to extraction. In the final extraction protocol, the carotenoids and chlorophylls were extracted twice with 500 µl diethyl ether: hexane (1:1). With each extraction the sample was vortexed for 30 min in a 2 ml micro-centrifuge tube after which it was centrifuged at 12 000 rpm for 2 min. The upper organic phase of each extraction was collected, pooled and dried under a stream of nitrogen. Dried samples were stored under a nitrogen atmosphere at -20ºC. Prior to RP-HPLC analysis, samples were dissolved in 200 µl of a 1:4 ethyl acetate, methanol solution containing 0.1% (w/v) BHT and centrifuged for 2 min at 12 000 rpm. Samples were shielded from strong light and kept on ice during all procedures.

Additionally, different solvents and extraction times were investigated. Acetone and diethyl ether:hexane (1:1) were tested as a possible extraction solvent comparing 5 min and 30 min extraction times.

The effects of normal laboratory light conditions (± 32 000 lumens) and pH on carotenoid and chlorophyll degradation during the extraction procedure were also investigated. The standard extraction procedure using subdued light conditions were compared with normal laboratory (±32 000 lumens fluorescent light) light conditions. The effect of pH, (since green berry tissue has a lower pH than red berry tissue), was evaluated by comparing the extraction procedure as mentioned above to an extraction procedure where the 500 µl millipore water added in the first step of extraction was replaced by 500 µl of a 50 mM Tris–HCl (pH 7.5) solution containing 1 M NaCl.

3.2.6 CHROMATOGRAPHIC CONDITIONS

The carotenoid and chlorophyll pigments were separated by RP-HPLC on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD system. An YMC30 column (250 mm x 4.6 mm; particle size 5µm) and YMC30 guard cartridge (10 mm x 4 mm, particle size 5 µm), both from YMC Europe (Schermbeck, Germany) were used. The C_{30} column has been shown to provide excellent resolution of photo-isomerised standards of various carotenoids (Emenhiser *et al.* 1996a, 1995). Chemstation software for LC3D (Rev.A.10.01[1635]; Hewlett-Packard, Waldborn, Germany) was used for data processing.

RP-HPLC chromatography conditions similar to Taylor *et al.* (2006) were used with small alterations. Binary solvents consisting of 3% ddH_2O in methanol containing 0.05 M ammonium acetate (Solvent A) and 100% MTBE (Solvent B) were used, where both solvents contained 0.1% (w/v) triethylamine. A flow rate of 1 ml/min was used at 25 ºC with an injection volume of 20 µl. Elution was according to the following program: isocratic at 20% B for 20 min followed by a linear gradient from 20% B to 50% B in 4 min, isocratic at 50% B for 4 min followed by a linear increase to 68% B in 2 min, isocratic at 68% B for 2 min followed by a linear decrease to 20% B. The column was equilibrated for 15 min at the starting conditions before each injection.

3.2.7 IDENTIFICATION AND QUANTIFICATION OF CAROTENOIDS

Identification of carotenoids and chlorophylls in Merlot grape samples was achieved by comparing retention times and visible spectra with authentic standards and published literature (De Rossa and Mercadante 2007; Dugo *et al.* 2008; Mendes-Pinto *et al.* 2005; Taylor *et al.* 2006; Van Breemen *et al*. 1991). The elution of the various carotenoid and chlorophyll pigments was followed at 420 nm, 450 nm and 470 nm with a constant reference wavelength at 800 nm (Taylor *et al.* 2006). Standard curves for the quantification of carotenoids and chlorophylls were obtained by plotting amount (ng)

against area which was obtained by triplicate injections. Liquid chromatography mass spectrometry (LC-MS) was performed using a Waters API Q-TOF Ultima connected to a Waters UPLC (Waters Corporation, Massachusetts, USA) system. The same conditions used before were employed except the mobile phases were slightly changed. Solvent A was 3% ddH_2O in methanol and Solvent B was 100% MTBE. The chlorophylls and carotenoids were detected with atmospheric pressure chemical ionization (APCI) system in the positive mode. A cone voltage of 35 V was used with the Q-TOF Ultima MS system. A capillary voltage of 3.5 kV with a desolvation temperature of 350ºC was also employed. The LC-MS analyses were performed to confirm identification of chlorophyll and carotenoid derivatives for which authentic standards were not available.

3.2.8 LIMIT OF DETECTION AND QUANTIFICATION

The LOD (limit of detection) was defined as the amount that results in a peak with a height three times that of the baseline noise. The LOQ (limit of quantification) was defined as the lowest injected amount which could be reproducibly quantified (STDEV ≤ 5%).

3.2.9 SELECTIVITY AND RECOVERY

The extraction method efficiency was evaluated by doing mock extractions with mixtures of the authentic standards. In a mock extraction the extraction protocol is followed except that no grape tissue is present in the matrix. Recovery of individual carotenoids from the sample matrix was determined according to the amount extracted from the matrix spiked with known concentration of a mix of authentic standards minus the extract from the matrix alone. The normal extraction protocol was followed for all the samples.

3.3 RESULTS AND DISCUSSION

3.3.1 IDENTIFICATION AND QUANTIFICATION OF CAROTENOIDS AND CHLOROPHYLLS IN GRAPE BERRIES

The following carotenoids could be separated and identified by RP-HPLC comparing spectra and retention times of cv. Merlot grape sample peaks with those of authentic standards: neoxanthin, lutein, chlorophyll b, zeaxanthin, chlorophyll a and β-carotene. In Figure 3.1 the HPLC chromatograms of red and green tissue are shown. It was observed that chlorophyll derivatives and degradation products, particularly in the green berry tissue, were present in fairly high amounts. These derivatives and degradation

products were identified as chlorophyllide a, chlorophyll a', pheophytin a, pyropheophorbide b, chlorophyll b', pheophorbide b, pyropheophytin b and pheophytin b in comparison with authentic standards (chl a and b), elution time, spectra according to literature (Canjura and Schwartz 1991; Van Breemen *et al*. 1991) and molecular masses obtained by MS (Table 3.2). Unknown carotenoid-like compounds were identified in comparison with the literature (De Rossa and Mercadante 2007; Faria *et al*. 2009; Zepka and Mercadante 2009) and LC-MS as *cis*-violaxanthin, neochrome, *cis*-neoxanthin, luteoxanthin, flavoxanthin, auroxanthin, mutatoxanthin, cis-β-carotene and 5,8-poxy-β-carotene (Table 3.2). 5,8-Epoxy-β-carotene was identified according to its elution time, maximum absorbance and fine structure in the methanol/MTBE mobile phase (De Rosso and Mercadante, 2007; Zepka and Mercadante 2009). *Cis*-neoxanthin was identified by the hypsochromic shift of 18 nm compared to all-*trans*-neoxanthin and the high intensity of the *cis* peak (Britton *et al*. 1995) (Table 3.2). Similarly cis-violaxanthin was identified by the hypsochromic shift of 8 nm and the intensity of the *cis* peak (Britton *et al.* 1995). The *cis*-isomer of β-carotene was identified by comparison to literature (De Rosso and Mercadante, 2007; Faria et al. 2009; Zepka and Mercadante, 2009) retention time and taking into account the hypsochromic shift of 6 nm and increased intensity of the *cis*-peak ($\%A_B/A_{II}$) (Britton *et al.* 1995) as well as by MS (Table 3.2). Mutatoxanthin was identified according to absorbance and retention time in similar mobile phase separations (De Rosso and Mercadante, 2007, Zepka and Mercadante, 2009;) and its molecular ion and fragment ions by MS (Table 3.2). Luteoxanthin and auroxanthin were identified according to their spectra and formation from violaxanthin when acidified with 0.1M HCl (Mínguez-Mosquera and Gandul-Rogas, 1994). Violaxanthin has two 5,6-epoxide groups in its molecule, which can transform at low pH to luteoxanthin with one 5,6-epoxide and one 5,8-furanoid group. Finally, both of these gave rise to the isomer auroxanthin. Similarly, neoxanthin changed into mutatoxanthin with one 5,8-furanoid and then neochrome with two 5,8-furanoid groups under low pH conditions.

Other breakdown products were also observed, but in smaller quantities, including chlorophyllide a, chlorophyll a' and pyropheophytin (Figure 3.2). Chlorophyllides are formed when the phytyl group of the chlorophyll is cleaved. This is usually catalyzed enzymatically by the endogenous enzyme, chlorophyllase. Chlorophyll a' is formed through epimerization of the C-10 centre of the chlorophyll. Several studies have shown that heating causes isomerisation of chlorophyll (Schwartz et al. 1981). Pyropheophytin is formed through decarbomethoxylation of the C-10 centre of pheophytin (Figure 3.2).

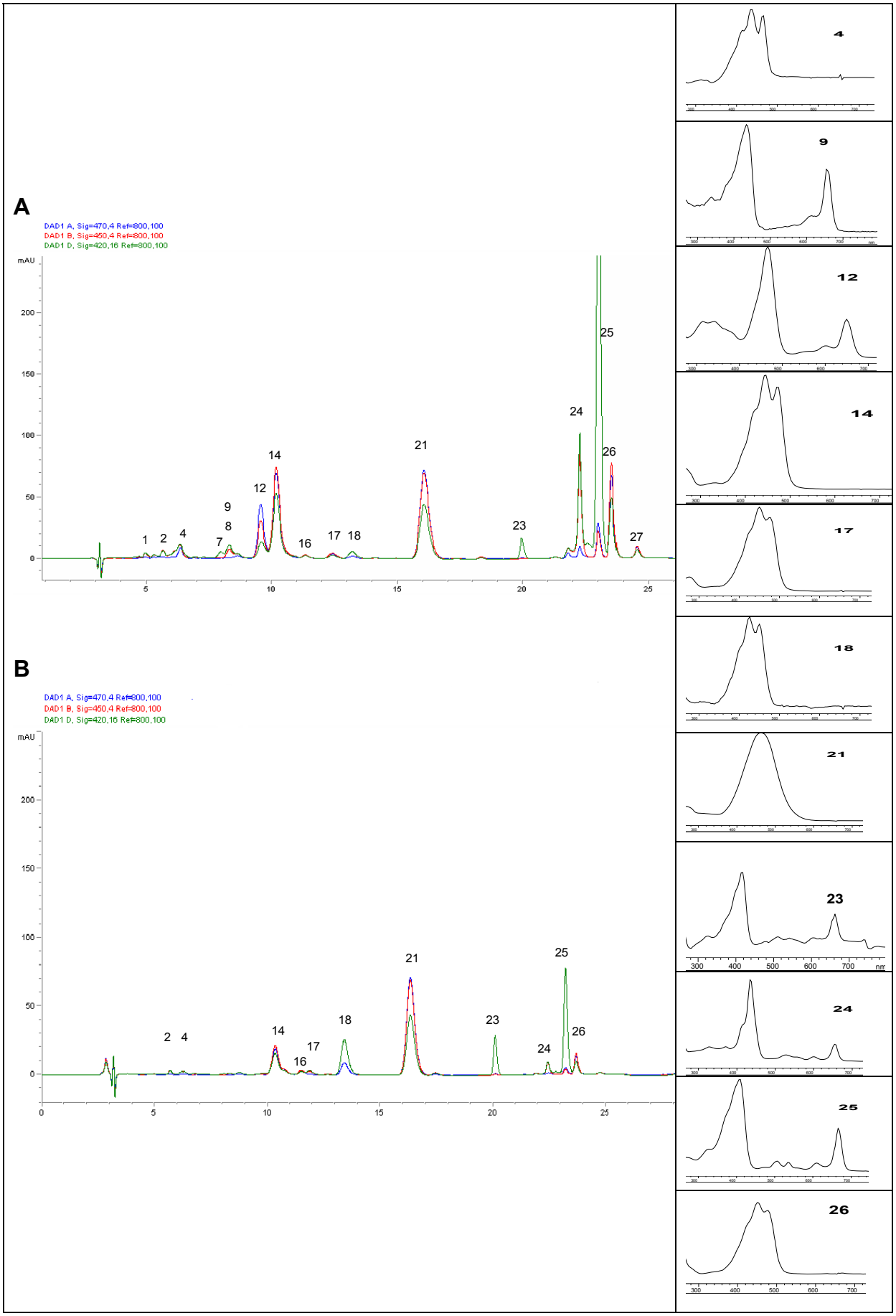


Figure 3.1 RP-HPLC profiles of the major carotenoids and chlorophylls in Merlot grape pre-veraison (A) and post-veraison (B) berries (1) *cis*-violaxanthin; (2) neochrome; (4) *cis*-neoxanthin; (7) luteoxanthin; (8) chlorophyllide a (9) pyropheophorbide b; (12) chlorophyll b; (14) lutein; (16) mutatoxanthin; (17)

zeaxanthin (18) 5,8-epoxy-β-carotene (21) β-apo-caroten-8-al; (23) pyropheophytin b; (24) pheophytin b; (25) pheophytin a; (26) β-carotene; (27) 9-*cis*-β-carotene.

Table 3.2 Peak identification of grape carotenoids on a C30 RP-HPLC column.

Peak nr	Compound	t_R(min)	A_B	Absorbance			% (III/II)	% (A_B/II)	APCI-MS		Identification
				I	II	III			[M+H]	Fragmentation ions (m/z)	
1	*Cis*-violaxanthin	5	320	410	430	458	44.7	17			t_R, Spectra
2	Neochrome	5.7		398	422	450	80.3			nd	t_R, Spectra
3	Violaxanthin	5.9		418	438	470	83.4		601.4	583.4 [M+H-18]	MS, t_R, Spectra
4	*Cis*-neoxanthin	6.2	314	398	418	442	66	19		nd	t_R, Spectra
5	Neoxanthin	6.5		412	436	464	93.9		601.4	583.4 [M+H-18]	MS, t_R, Spectra
6	Neochrome	6.9		398	422	450				nd	t_R, Spectra
7	Luteoxanthin	8.2		400	422	441	100		601.4	nd	t_R, Spectra
8	Chlorophyllide a	8.3		430	658				615.5	nd	t_R, Spectra
9	Pyropheophorbide b	8.4		434	658						t_R, Spectra
10	Flavoxanthin	8.9		402	424	451	71.1		585.9	nd	t_R, Spectra
11	Neochrome	9.3		398	422	450	87				t_R, Spectra
12	Chlorophyll b	9.6		466	650				907.5	nd	t_R, Spectra
13	Auroxanthin	10		382	402	426	99				t_R, Spectra
14	Lutein	10.3		422	446	474	57.6		569	551.4 [M+H-18], 533 [M+H-18-18]	MS, t_R, Spectra
15	Chlorophyll b'	11.1		466	650				907.5	nd	
16	Mutatoxanthin	11.9	310	398	418	442	30.8	22.5	585	567.4 [M+H-18], 549.4 [M+H-18-18]	MS, t_R, Spectra
17	Zeaxanthin	12.4		422	450	478	22.4		569.4	551.4 [M+H-18]	MS, t_R, Spectra
18	5,8-epoxy-B-carotene	13.2		402	426	450	49.2			nd	t_R, Spectra
19	Pheophorbide b	14							607.4	nd	t_R, Spectra
20	Chlorophyll a	15.8		430.8	666				893.5	nd	t_R, Spectra
21	β-apo-caroten-8-al	16.2			460				417.0	nd	MS, t_R, Spectra
22	Chlorophyll a'	17.5		430.8	666				893.5	nd	nd
23	Pyropheophytin b	20.1		418	662				827	nd	t_R, Spectra
24	Pheophytin b	22.4		434	654				885.5	nd	t_R, Spectra
25	Pheophytin a	23.2		410	666				871.5	nd	t_R, Spectra
26	B-carotene	23.7		425	452	477	22.9		537.4	nd	t_R, Spectra
27	9-*cis*-β-carotene	24.7	342	422	446	470	22	13.4	537.4	nd	t_R, Spectra

The most common carotenoids that were found in Merlot grape extracts were β-carotene, 5,8-epoxy-β-carotene and lutein, representing almost 85% of the total amount of carotenoids accompanied by minor carotenoids like neoxanthin, violaxanthin, zeaxanthin, neochrome, flavoxanthin, luteoxanthin and cis-β-carotene (Appendix A, Table 1 and 2). Similar results were found by Baumes *et al.* (2002).

All of the abovementioned carotenoids and chlorophylls except 5,8-epoxy-β-carotene were previously reported to be found in grapes (Baumes *et al.* 2002; De Pinho

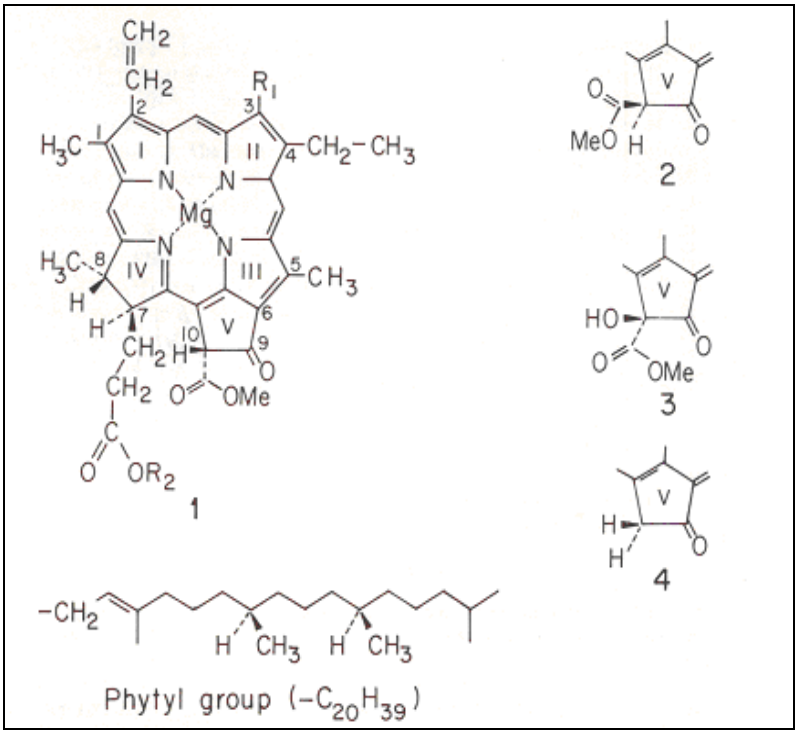
et al. 2001; Giovanelli and Brenna 2007; Mendes-Pinto *et al.* 2005; Oliveira *et al.* 2003, 2004; Razungles *et al.* 1988; Razungles *et al.* 1996). No literature on the carotenoid and chlorophyll content of Merlot grape berries could be found to date.

5,8-Epoxy-β-carotene and mutatoxanthin were quantified as zeaxanthin equivalents while *cis*-violaxanthin, *cis*-neoxanthin and neochrome were quantified as neoxanthin equivalents.

The LOD and LOQ of the carotenoids and chlorophylls for which authentic standards were obtained were determined and are shown in Table 3.3.

Table 3.3 Limit of detection and quantification of carotenoids and chlorophylls as determined by RP-HPLC.

Standards	LOQ mg/L	LOD mg/L
B-apocaroten-8-al	0.02	0.01
Antheraxanthin	0.05	0.02
B-carotene	0.10	0.01
Zeaxanthin	0.05	0.02
Violaxanthin	0.02	0.01
Neoxanthin	0.02	0.01
Lutein	0.10	0.02
Chlorophyll a	0.16	0.041
Chlorophyll b	0.09	0.02



Compound	Mg*	R1	R2	Isocyclic Ring (V)
Chlorophyll a	+	CH_3	Phytyl	1
Chlorophyll b	+	CHO	Phytyl	1
Chlorophyll a'	+	CH_3	Phytyl	2
Chlorophyll b'	+	CHO	Phytyl	2
Chlorophyllide a	+	CH_3	H	1
Chlorophyllide b	+	CHO	H	1
Pheophytin a	-	CH_3	Phytyl	1
Pheophytin b	-	CHO	Phytyl	1
Pheophorbide a	-	CH_3	H	1
Pheophorbide b	-	CHO	H	1
Chlorophyll a -1	+	CH_3	Phytyl	3
Pyropheophytin a	-	CH_3	Phytyl	4

***** In pheophytins and pheophorbides, Mg is replaced by 2H.

Figure 3.2 Structural formulas and nomenclature of chlorophyll a and b and their various derivatives adapted from Gross (1991).

3.3.2 EXTRACTION OF CAROTENOIDS AND CHLOROPHYLLS FROM GRAPE BERRIES

The selectivity and recovery of the RP-HPLC and extraction method were evaluated. The recoveries of all authentic standards were ≥79% from the mock extraction, which indicated that the extraction methodology was appropriate for the extractions of carotenoids and chlorophylls. The recovery of the authentic standards from red grape tissue was very good (≥77), except for violaxanthin (49%) (Table 3.4) but was improved to 63% when degradation products (*cis*-violaxanthin) were included. The recovery of chlorophyll a, chlorophyll b, violaxanthin and neoxanthin however, were very poor from

green berry tissue. This result was found to be mainly due to the low pH of the tissue which facilitates the degradation of chlorophyll a and b to pheophytin a and b respectively; and violaxanthin and neoxanthin respectively degraded to auroxanthin and luteoxanthin and neochrome and mutatoxanthin. *Cis*-violaxanthin and *cis*-neoxanthin were also formed from violaxanthin and neoxanthin respectively (Canjura and Schwartz 1991; Minguex-Mosquera and Gandul-Rojas 1994; Van Breemen *et al*. 1991). *Cis-trans* isomerisation has been shown to be mainly mediated by heat (Mínguez-Mosquera and Gandul-Rogas, 1994). When the pheophytin a and b and pyropheophytin b forms were included in recovery calculations, recovery improved to 67% for chlorophyll a and 113% for chlorophyll b. Poor recovery of both violaxanthin and neoxanthin from green tissue, resulted in unreliable quantification of these compounds and were therefore not quantified further in the experimental section (Chapter 4). The green and red berry tissues were investigated because it represented the extreme stages of development in the different grape tissues analysed. The differences in recovery between the green and red berry tissue were due to matrix differences, by which the pH differences between the tissue extracts would have made an important contribution to the recovery of pigments. There was a significant variance in recovery of compounds such as chlorophyll a, b and lutein between the red and green grape tissue matrix. This extraction method was, however, used to obtain a profile of the carotenoid and chlorophyll pigments in grape tissue of different maturities and was not optimized for the extraction of a specific compound in a specific grape matrix.

Table 3.4 Recovery of authentic standards.

Compound	[a]Mock extraction % Recovery*	[b]Mock extraction (*ISTD) % Recovery	[c]Green tissue % Recovery	Green tissue % Recovery without breakdown products	[d]Red tissue % Recovery	Red tissue % Recovery without breakdown products
Violaxanthin	89.0	101.0	0.0	0.0	63.2	49.1
Neoxanthin	79.4	90.1	55.9	22.4	97.6	95.0
Chlorophyll b	109.2	124.0	113.4	0.0	78.4	86.5
Lutein	89.9	102.0	66.8	66.8	99.5	99.5
Zeaxanthin	92.9	105.4	99.0	98.9	95.6	95.6
Chlorophyll a	97.0	110.0	67.3	0.0	83.2	78.0
β-apo-carotenol-8-al (ISTD)	85.1	100.0	100.0	100.0	100.0	100.0
β-carotene	88.8	100.8	76.2	76.2	81.9	81.9

*Losses were compensated for according to internal standard (ISTD) in all cases, except in this column.
[a]Mock extraction: on authentic standards without compensation according to ISTD, [b]Mock extraction: extraction of authentic standards, [c]Green tissue: extraction of authentic standards together with green lyophilised berry tissue recovery % includes all breakdown products: violaxanthin (sum of violaxanthin and *cis*-violaxanthin), neoxanthin (sum of neoxanthin and neochromes), chlorophyll b (sum of chlorophyll b, pheophytin b and pyropheophytin b), lutein (lutein), zeaxanthin (zeaxanthin), chlorophyll a (sum of chlorophyll a and pheophytin a), β-carotene (β-carotene). [d]Red tissue: extraction of authentic standards together with red lyophilised berry tissue recovery % includes all breakdown products: violaxanthin (sum of violaxanthin and *cis*-violaxanthin), neoxanthin (sum of neoxanthin and neochromes), chlorophyll b (sum of chlorophyll b, pheophytin b and pyropheophytin b), lutein (lutein), zeaxanthin (zeaxanthin), chlorophyll a (sum of chlorophyll a and pheophytin a), β-carotene (β-carotene) All values are the average of 4 replicates.

3.3.3 INVESTIGATION OF EXTRACTION SOLVENTS, SAMPLE PROCESSING AND STORAGE

Acetone is a common solvent mentioned in literature used to extract chlorophylls (Mangos and Berger 1997; Lichtenthaler and Wellburn 1983; Hemraj *et al.* 1997) and carotenoids (During and Davtyan 2002; Steel and Keller 2000) from leaves and various food types. Mendes-Pinto *et al.* (2004) found that a mixture of hexane/diethyl ether 50/50 was the most effective for extracting both neoxanthin and β-carotene from grape berry tissue which are important aroma precursors in wine (Mendes-Pinto *et al.* 2004). These two solvents were evaluated as potential extract solvents for extracting both chlorophylls and carotenoids from grape tissue (Table 3.5 and 3.6). Hemraj *et al*. (1997) found that the amount of chlorophyll extracted is influenced by how finely the plant sample was ground and on the length of extraction time in the acetone. The longer the extraction time, the more time the acetone has to break the protein complex and remove the chlorophyll pigments. Thus an extraction time of 5 min and 30 min were also investigated (Table 3.5 and 3.6). This experiment was conducted as mock extractions with authentic standards.

Carotenoids and chlorophylls were found to be more stable in diethyl ether:hexane (1:1) than in acetone since less degradation products of carotenoids and chlorophylls were found when a 30 min extraction period was used. A 30 min extraction period

increased the extraction of most carotenoids without an increase in degradation products and was chosen as the optimal extraction time (Table 3.5 and 3.6).

Table 3.5 The efficiency of ethylether:hexane (1:1) as extraction solvent for carotenoids and chlorophylls in lyophilised grape tissue during two different extraction times.

Compound	Ethylether:hexane			
	30 min extraction		5 min extraction	
	Amount recovered (ng)	% Recovery	Amount recovered (ng)	% Recovery
Violaxanthin	58.62 ± 1.59	100.97	59.50 ± 1.03	102.48
Neoxanthin	54.48 ± 1.72	95.61	55.17 ± 2.55	96.83
Antheraxanthin	84.07 ± 2.01	100.39	84.87 ± 0.50	101.83
Chlorophyll b	244.55 ± 6.62	101.45	247.44 ± 0.99	102.65
Lutein	168.93 ± 4.67	102.70	168.61 ± 1.05	102.50
Zeaxanthin	69.22 ± 2.93	102.40	68.83 ± 1.21	101.82
Chlorophyll a	129.00 ± 4.90	102.58	129.58 ± 3.55	103.04
β-carotene	117.45 ± 1.90	122.89	114.56 ± 0.69	119.87

Amount recovered was calculated as the average of 3 replications.

Table 3.6 The efficiency of acetone as extraction solvent for carotenoids and chlorophylls in lyophilised grape tissue during two different extraction times.

Compound	Acetone			
	30 min extraction		5 min extraction	
	Amount recovered (ng)	% Recovery	Amount recovered (ng)	% Recovery
Violaxanthin	56.73 ± 0.46	97.72	55.45 ± 0.52	95.51
Neoxanthin	53.62 ± 0.29	94.11	53.02 ± 0.38	93.06
Antheraxanthin	81.79 ± 1.28	97.67	79.24 ± 1.03	94.62
Chlorophyll b	235.81 ± 3.36	97.83	228.52 ± 4.98	94.80
Lutein	163.55 ± 1.92	99.43	154.77 ± 2.47	94.09
Zeaxanthin	62.73 ± 0.74	92.80	60.96 ± 1.08	90.17
Chlorophyll a	125.78 ± 2.08	100.02	123.86 ± 0.55	98.49
B-carotene	119.37 ± 1.02	124.91	115.75 ± 1.60	121.12

Amount recovered was calculated as the average of 3 replications.

Lyophilisation of plant tissue is a well known practice to preserve plant tissue samples and has been used widely to preserve grape tissue samples for the evaluation of carotenoid content (During and Davtyan 2002; De Pinho *et al*. 2001, Razungles *et al.* 1988; Steel and Keller 2000). Craft *et al.* (1993) reported in his work that the hydrocarbon carotenoids (carotenes) showed some degradation and xanthophylls increased when tissue was lyophilised which might be due to the more efficient hydrolysis of xanthophyll esters. Degradation of carotenoids in vegetables during lyophilisation was also reported by Park (1987). We suggest that degradation of chlorophyll is also possible during lyophilisation since the water is removed from the tissue concentrating the acid in the matrix which might facilitate chlorophyll degradation.

Van den Berg *et al*. suggested, in his review on the potential of improvement in the carotenoid levels in food, the storage of food samples at -20ºC and for long term storage at a temperature of -70ºC. Craft *et al.* (1988) reported that carotenoids in serum samples stored at -70ºC were stable for at least 2 years. Van den Berg *et al.* (2000) also recommended that when samples are stored for long periods before analyses, it is necessary to store samples together with reference samples from which the carotenoid content is known to compensate for degradation losses and to identify breakdown products easily. We found that dried aliquots of standards, especially chlorophyll a and violaxanthin was unstable and almost immediately started degrading. For this reason samples were not stored for longer then 48 hours at -20ºC.

3.3.4 EFFECT OF pH AND LIGHT ON EXTRACTION EFFICIENCY

The effect of light and pH (respectively) during the extraction method used in this study was evaluated by adding a buffer solution which replaced the water in the extraction method (50 mM Tris-HCl 7.5 pH containing 1 M NaCl) and working under subdued light conditions instead of normal laboratory light conditions (Table 3.7). The effect on carotenoids and chlorophylls were calculated with and without their degradation products.

More chlorophyll b and neoxanthin were recovered from green tissue in the presence of the buffer (Table 3.8) although lutein, β-carotene, and zeaxanthin were recovered in lower amounts. Moreover in the red tissue, chlorophyll a, chlorophyll b and zeaxanthin showed higher recovery while lutein, β-carotene, and neoxanthin were recovered in lower amounts. In the case of the green tissue where the chlorophylls and carotenoids during the extraction process and analysis were protected from light higher recovery of all the carotenoids and chlorophylls were evident (Table 3.7). Almost a 30% increase in the extraction of lutein, zeaxanthin and β-carotene were found in green tissue under subdued light conditions compared to normal laboratory light conditions during extraction. For the red tissue subdued light only improved the recovery of neoxanthin, chlorophyll b and β-carotene (Table 3.7). Working under subdued light conditions is a common practice when working with carotenoids and chlorophylls and is suggested to prevent *cis/trans* isomerisation and degradation (Van den Berg *et al.* 2000).

Table 3.7 The effect of light on the extraction of carotenoids and chlorophylls (pigments) from red and green berry tissue.

Compound	% More pigments without light exposure			
	Red tissue		Green tissue	
	Without breakdown products	[a]With breakdown products	Without breakdown products	[a]With breakdown products
Violaxanthin	0.0		0.0	
Neoxanthin	0.0	15.8	0.0	15.8
Chlorophyll b	-7.2	31.8	-7.2	31.8
Lutein	31.5	31.5	31.5	31.5
Zeaxanthin	33.3	33.3	33.3	33.3
Chlorophyll a	0.0	29.8	0.0	29.8
β-carotene	30.6	30.6	30.6	30.6

[a]With breakdown products: recovery % includes all breakdown products: violaxanthin (sum of violaxanthin and *cis*-violaxanthin), neoxanthin (sum of neoxanthin and neochromes), chlorophyll b (sum of chlorophyll b, pheophytin b and pyropheophytin b), lutein (lutein), zeaxanthin (zeaxanthin), chlorophyll a (sum of chlorophyll a and pheophytin a), β-carotene (β-carotene).
All values are the average of 4 replicates.

Table 3.8 The effect of pH on the extraction of carotenoids and chlorophylls (pigments) from red and green berry tissue.

Compound	% More pigments with buffer			
	Red tissue		Green tissue	
	[a]Without breakdown products	With breakdown products	[a]Without breakdown products	With breakdown products
Violaxanthin	0.0	0.0	0.0	0.0
Neoxanthin	-17.3	-81.8	100.0	-18.8
Chlorophyll b	40.2	18.6	5.4	-32.5
Lutein	-15.1	-15.1	-26.3	-26.3
Zeaxanthin	19.8	19.8	-29.1	-29.1
Chlorophyll a	100.0	-13.0	0.0	-34.6
B-carotene	-23.7	-23.7	-60.7	-26.7

[a]With breakdown products: recovery % includes all breakdown products: violaxanthin (sum of violaxanthin and *cis*-violaxanthin), neoxanthin (sum of neoxanthin and neochromes), chlorophyll b (sum of chlorophyll b, pheophytin b and pyropheophytin b), lutein (lutein), zeaxanthin (zeaxanthin), chlorophyll a (sum of chlorophyll a and pheophytin a), β-carotene (β-carotene).
All values are the average of 4 replicates.
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It is evident that the low pH of the berries especially green berries (pH < 3.15) compared to red berries (pH ≥ 3.5) facilitated the transition of chlorophyll a and b to pheophytin a and b. The pheophytins are formed when the central Mg atom of the chlorophyll are replaced with a hydrogen ion (Schwartz and Lorenzo 1990), especially in the presence of plant acids from the vacuoles of extracted plant material (Schwartz *et al.* 1981; Ferruzzi and Schwartz 2005). The addition of salts during grinding of tissue has been recommended to prevent the formation of pheophytins, especially in plants with acidic cytoplasm. However Strain *et al.* (1971) has found that addition of neither

$CaCO_3$ nor $MgCO_3$ could totally prevent the formation of pheophytins in the extraction of chlorophyll from acidic tissue. In green tissue, even when extracted in the presence of a buffer, all the chlorophyll a was already converted to pheophytin a, which indicates that degradation already took place during lyophilisation of tissue and/or during storage (Table 3.8). In the red berry tissue extracts, there were also pheophytins present even when it was protected against the pH effect during extraction (Table 3.8). The amounts present in red tissue were however much less compared to green tissue. It is evident that although the addition of a 50 mM Tris–HCl (pH 7.5) buffer to the extraction solvent decreased the formation of pheophytins significantly, it also decreased the extraction of carotenoids. The percentage increase of chlorophyll extraction in the presence of the buffer determined with breakdown products included was actually negative, because the formation and extraction of the breakdown products decreased significantly in the presence of the buffer. In the lyophilized green tissue there was no chlorophyll a present and the buffer could thus only influence the extraction of pheophytin a, not its formation. In the green berry tissue only 25% less pheophytin a and b were formed with the addition of the buffer during extraction, while in the red berry tissue 184 and 86% less pheophytin a and b were respectively formed. This indicates that the buffer was not strong enough to neutralize the acid in the green tissue.

Although the extraction method used in this study was similar to those used by other authors (Mendes-Pinto *et al.* 2004; Oliveira *et al.* 2003) for grape berries, it is clear that it should be further optimized for the extraction of both carotenoids and chlorophylls and to minimize the effect of pH during extraction. Razungles *et al.* (1996) mentioned the addition of 3 g of magnesium hydroxyl carbonate to the homogenate of mature berries and 6 g to green berries during extraction in his study on carotenoids during maturation of grape berries. Razungles *et al.* (1996) did not identify or report any *cis*-isomers of carotenoids, but also did not include the evaluation of chlorophyll in grape berries.

Another study reporting the use of a buffer during carotenoid extraction is Dias *et al.* (2009) on Portuguese fruit and vegetables. The addition of sodium, magnesium or calcium carbonate (0.10 g per gram of sample) to neutralize acids in tissue samples when extracting carotenoids have been suggested to avoid *cis/trans* isomeration (Mangels *et al.* 1993; Van den Berg *et al*. 2000; Zakaria *et al*. 1979).

It is interesting to note that changes in pH within the thylakoid membrane (where carotenoids and chlorophylls are located) facilitate these typical biochemical conversions in the xanthophyll cycle (Demmig-Adams *et al.* 1996).

3.4 CONCLUSION

The RP-HPLC method baseline separated all the carotenoids and chlorophylls and their derivatives. Recovery of standards from mock extractions was high, indicating that the extraction procedure was acceptable. However, it is clear that when the extraction recovery of the standards were tested in the matrix of the grape tissue the situation is less promising due to the high acid content of grape tissue. Violaxanthin, neoxanthin and the chlorophylls were especially sensitive to low pH conditions which facilitated their degradation. The degradation products of these compounds under acidic conditions were identified as pheophytin a, b, chlorophillide a, pyropheophytin b, *cis*-violaxanthin, *cis*-neoxanthin, neochrome, mutatoxanthin and luteoxanthin. There is a possibility that some degradation products were already present in the tissue due to lyophilisation (since the water in the berry was then removed and the acid concentrated). More work is needed to investigate the effect of lyophilisation and storage on the composition of grape tissue of different maturity. The extraction method for grape berry tissue at different ripening stages should also be optimised further too effectively neutralise tissue acidity, without compromising the extraction of carotenoids significantly, in especially green berry tissue. The question as to whether *cis*-isomers and chlorophyll degradation products are naturally present in grape berries or are formed during sampling and processing remains unanswered in the current study.

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Chapter 4

RESEARCH RESULTS

Quantitative analysis of grape carotenoid and chlorophyll profiles during ripening with reference to grapevine vigour and water status

Research Results

4.1 INTRODUCTION

Research has shown that carotenoids are the likely precursors to C_{13}-norisoprenoids which is thought to be a significant group of aroma compounds in wine due to their low olfactory threshold values (Etievant *et al.* 1991). Photosynthetic pigments such as carotenoids and chlorophylls, and their derivatives, are also reported to be found in wine (De Pinho *et al.* 2001) and have the potential to contribute as precursors to aroma compounds (Sefton *et al.* 1993).

One of the important C_{13}-norisoprenoids which contributes to wine aroma is β-ionone with a low threshold value of 90 ng/L (in a model base wine) (Kotseridis *et al.* 1999b). β-ionone has a violet-like aroma and can be formed as a cleavage product of the carotenoid β-carotene (Kanasawud and Cruzet 1990) and zeaxanthin, a xanthophyll (Mathieu *et al.* 2005). β-damascenone is another C_{13}-norisoprenoid found in wine, with a threshold value of 50 ng/L in 10% alcohol (Guth 1997), its aroma notes have been described as honey-like (Kovats 1987) and flowery, ionone-like (Ohloff 1978). Due to the fact that the precursors to these aroma compounds are grape-derived, they are an important research field for viticulturists, since they can potentially be altered by viticultural practices and influence the perception of the end product.

Viticultural factors which can influence carotenoid and chlorophyll content in grapes are, for example, sunlight and plant water deficit (Bindon 2004; Bureau *et al.* 1998; Bureau *et al*. 2000; Oliveira *et al*. 2003; Oliveira *et al.* 2004; Razungles *et al.* 1998). These factors can be managed by viticulturists through the manipulation of vigour through canopy management and irrigation scheduling, giving an opportunity for viticultural research. Additionally, the advance in technology to develop non-invasive techniques to monitor vineyard progression in ripening, for example via chlorophyll measurements (Kolb *et al.* 2006) creates further possibilities for the viticulturist. Kolb *et al*. (2006) found that non-invasive chlorophyll fluorescence measurements are well suited to determine sugar accumulation in grape berries during ripening. Since pigmentation has been demonstrated to be a statistically significant indicator of transcriptional state of genes during the initiation of ripening (Lund *et al.* 2008), the non-invasive monitoring of grape pigments can provide the viticulturist with information regarding the timing of key metabolic events which can be

used to predict optimal ripeness. Given that grape pigments, namely carotenoids and chlorophylls, respond sensitively to metabolic events and grapevine physiology, the possibility exists that the monitoring of these pigments can be used in building a within-vineyard model to predict ripeness using non-invasive measurements such as near infra-red (NIR) radiation or chlorophyll fluorescence (Agati *et al.* 2008; Baranska *et al*. 2006; Davey *et al*. 2009; Kolb *et al*. 2006; Ruiz *et al.* 2008).

However, before research is initiated to develop non-invasive methods for pigment monitoring in the vineyard, two key questions need to be addressed. Firstly, a reliable analytical method is required for validation and calibration of the non-invasive measure (Agati *et al*. 2007; Gitelson and Merzlyak 2002). Secondly, the factors influencing these pigment concentrations need to be evaluated in greater depth. These factors are diverse, and include temperature, soil water content, sunlight penetration, cultivar, terroir, clone and climate (Bindon 2004; Bindon *et al.* 2007; Bureau *et al.* 1998; Bureau *et al*. 2000; Giovanelli and Brenna 2007; Marais *et al*. 1992a; Oliveira *et al.* 2003; Oliveira *et al*. 2004; Oliveira *et al*. 2006; Razungles *et al.* 1998). However, for regional or localised vineyards, small variations in grapevine vigour, microclimate or soil type can alter the timing of phenology, and thus harvest. On a small scale, the monitoring of pigment changes during grape development can be determined relative to other ripeness parameters e.g. sugars, organic acids and colour in order to explore these relationships statistically. To date, little work has been done on carotenoids and chlorophylls in relation to the above-mentioned ripeness parameters in grapes.

Chemometrics is a valuable tool to explore relationships of grape composition and the viticultural parameters which can potentially affect them since many variables can be accommodated in one analysis. Chemometric techniques that can be used to discriminate between samples and explore potential relationships are principle component analyses (PCA) and partial least square (PLS) analysis. PCA and PLS analysis describe sample clustering and detect compounds responsible for the separation of samples (Kemsley 1998). PCA is essentially a descriptive method used to visualise samples present in an n-dimensional space of a set of inter-correlated variables into a smaller number of dimensions, called principle components (PCs), where each principle component (PC) account for a portion of the total variance of the data set (Kemsley 1996; Summer *et al*. 2003). PLS is based on multivariate regression, taking into account the covariance between variables. PLS is regularly used to predict quantitative variables using

spectroscopic measurements. These techniques form the starting point for an exploratory study, and can serve as a guideline to test the validity of hypotheses using large datasets, as well as the foundation to generate statistical models.

In one study, Pereira *et al.* (2006) showed by using ^{1}H NMR spectroscopy (fingerprinting of metabolites in grape skins) together with chemometric data analyses that vintage effects on grape metabolic profiles prevail over soil effect. In a later study, Le Moigne *et al.* (2008) showed that front face fluorescence spectroscopy and visible spectroscopy coupled with chemometrics had the potential to characterise ripening of Cabernet Franc grapes. For the viticultural scientist, the limitations of this type of study are that models can be built only with data from multiple seasons, and tested with data from diverse regions. To generate this type of data requires the use of rapid, non-invasive field measurements. As a preliminary study, this project has undertaken a study of the chlorophyll and carotenoid concentration of Merlot grape berries within a single vineyard. Using defined plots from variable regions within the vineyard, the vigour and soil water content of each plot was monitored in order to potentially correlate changes in grape pigments to viticultural parameters using PCA analysis. As a second question, the general trends of individual carotenoids and chlorophylls from pre-veraison to harvest are shown with reference to possible influence of seasonal variation on these profiles. Finally, some preliminary work has been done using PLS analysis, on the relationship between carotenoid and chlorophyll content and selected ripening parameters.

4.2 MATERIALS AND METHODS

4.2.1 PLANT MATERIAL AND GROWTH CONDITIONS

A commercial vineyard *Merlot Clone MO 9 vines* (*Vitis vinifera* L. *cv.* Merlot*)* grafted on Richter 110 (*Vitis berlandieri* x *Vitis rupestris*) rootstocks was used in this experiment. The vineyard is situated in the Stellenbosch region, South Africa on the Dornier wine estate 181-188 m above sea level with a slope of 12.1% over a distance of 174 m south to north. The area has a Mediterranean climate with dry and warm summers and cold, rainy winters. The annual rainfall for the Stellenbosch region is between 600 to 800 mm and the average temperature 18 to 19ºC. The vine spacing was 2.7 x 1.5 m in an east-west direction on a 6 wire movable hedge trellis system and planted in oakleaf soil. Sporadic occurrences of leaf roll virus and *Eutypa dieback (Eutypa lata)* disease were found at the vineyard site.

However, these vines were excluded from the experimental plots. No other diseases or rot has been detected. The vineyard is surrounded by mountains on the north and north-east side causing reduced sunshine hours in the morning. Canopy management included shoot positioning and mechanical shoot topping on some plots are indicated in Appendix B, Table 3a.

4.2.2 PLOT DESCRIPTION AND LAYOUT

The plot layout was based on normalized difference vegetation index (NDVI) images taken in January of each season (2006/2007 and 2007/2008). These images were used to select regions of vigour variability in the experimental vineyard. The NDVI images were based on the reflectance ratio between the far red and near infrared radiation reflected by the plant. Previous research has shown that the NDVI index is well correlated with biomass and pruning mass (Tucker 1979; Asrar *et al.* 1984; Daughtry *et al*. 1992; Johnson *et al.* 2001b; Nemani *et al*. 2001). Various researchers have reported positive correlations between pruning mass and vigour level (Johnson *et al*. 2001a; Johnson *et al.* 1996; Baldy *et al.* 1996a). High (blue), medium (green) and low (white) vigour areas were identified using the NDVI images and the plot layout was chosen accordingly (Figure 4.1 and 4.2). High, medium and low vigour plots each received two irrigation treatments, namely dry land (minimal to no irrigation) or wet (irrigated) (Appendix B, Table 1 and 2). The selected plots consisted of 24 vines each which were subdivided into 4 subplots of 6 vines each (Figure 4.3).

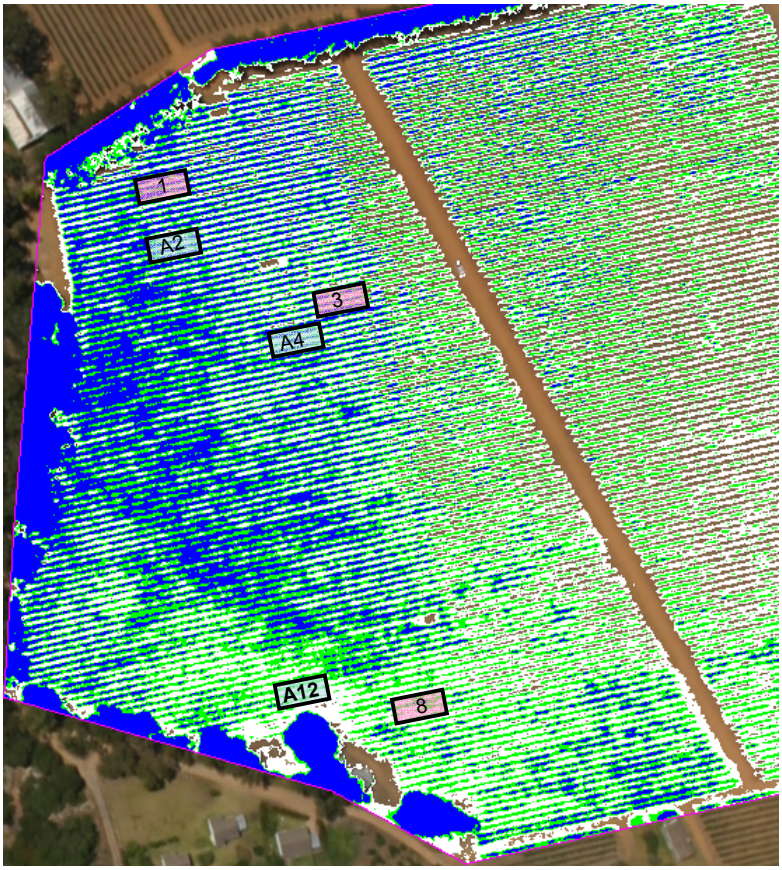

Figure 4.1 NDVI image of the experimental vineyard 2006/2007 season with plot layout: 1 (high vigour, dry); A2 (high vigour, irrigated); 3 (medium vigour, wet); A4 (medium vigour, dry); 8 (low vigour, dry); A12 (low vigour, wet).

Figure 4.2 NDVI image of the experimental vineyard 2007/2008 season with plot layout: 5 (high vigour dry); A9 (high vigour, wet); 3 (medium vigour, dry); A3 (medium vigour, wet); 2 (low vigour, dry); 8 (low vigour, dry); A12 (low vigour, wet).

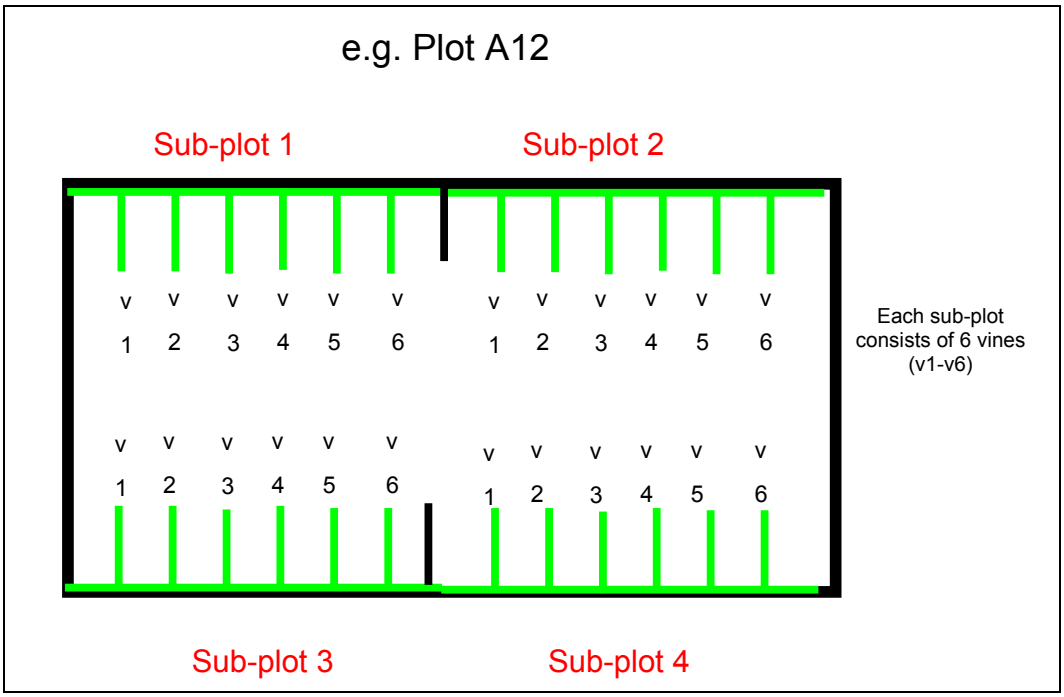

Figure 4.3 Diagram of a plot with four sub-plots of six vines each.

4.2.3 CLIMATIC MEASUREMENTS

In the first year of the study (2006/2007 season) temperature loggers were installed in the bunch zone of treatment grapevines. Data from the temperature loggers in the vineyard were compared with the weather station data on the farm. The data was well correlated with the weather station data for the site, when average daily temperature data from different temperature loggers were compared (Figure 4.4). For the following season, general temperature data for the 2007/2008 season for the site was collected from the weather station. The weather station provided hourly data on relative humidity, dry temperature, soil temperature, wind speed, rainfall and solar radiation which were converted to day (06h00 to 20h00) and night (20h00 to 06h00) averages (Appendix B, Table 3).

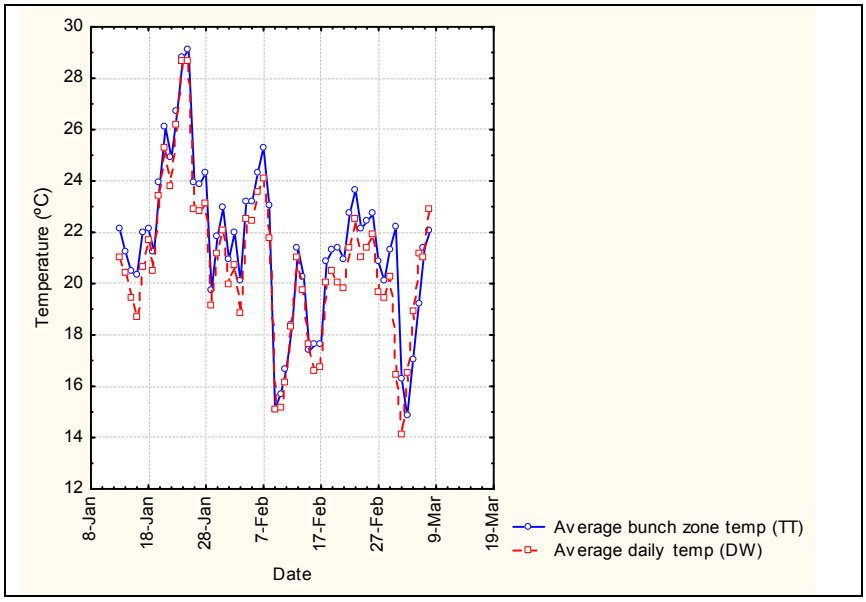

Figure 4.4 Average daily temperature measured by Dornier weather station (DW) compared to the average bunch zone temperature measured by tiny tag (TT) loggers installed in the bunch zone of a vine canopy for the 2006/2007 growing season.

4.2.4 CANOPY MEASUREMENTS

Photosynthetically active radiation (PAR) within the grapevine canopy was measured with a sun fleck ceptometer (Decagon Device, Inc. Pullman, Washington) for the 2007/2008 season. Measurements were taken post-veraison (29 Feb 08) on a cloudless day at solar noon between 09h00 and 10h00 in the morning. The ceptometer was placed horizontally in the bunch zone of the canopy to obtain measurements representative of the PAR reaching the bunches. PAR measurements were adjusted according to ambient light conditions (measured at each plot) and expressed as a ratio of PAR of bunch zone: PAR ambient.

Shoot length was measured from representative shoots of each plot collected after pruning. Main and lateral shoot length were measured and the number of nodes and lateral shoots counted. Shoot diameter was measured at the base, middle and tip of each representative shoot respectively and expressed as the average of these three diameter measurements. The number of shoots on each vine was determined as well as the number of shoots on spur positions which were potential grape bearers. Furthermore, the pruning mass of each experimental vine was weighed (Appendix B, Table 3a).

4.2.5 VINE WATER STATUS MEASUREMENTS

Pre-dawn leaf water potential was measured weekly from veraison to harvest at 04h00 prior to an irrigation event on all experimental plots using a pressure chamber (PMS Instrument Co., Corvallis, Oregon) supplied with a compressed air cylinder (Scholander *et al.* 1965). Four young fully expanded leaves per plot were used for measurements. Leaves were cut and inserted into the pressure chamber. Measurement was within 15 sec after the leaf was cut.

Soil water content was measured for each plot at 30 cm, 60 cm and 90 cm depths with a calibrated neutron probe (Hydro probe, model 503DR, 130 So Buchanan Pacheco, CA USA) for both seasons of the study. An average of these three depths was used as indicator of the wetness of the soil expressed as neutron count ratios (Appendix B, Table 3b). The instrument was calibrated against a 200 L water barrel incorporating a PVC access tube similar to the field-installed ones. The neutron count ratio was determined from the field measured values divided by the water drum count average.

4.2.6 YIELD MEASUREMENTS, BUNCH AND BERRY MASS

Bunch mass and the number of bunches per vine was recorded for each plot at harvest from which average bunch mass per vine was determined. Bunch mass, berry mass and the number of berries per bunch were measured from twelve representative typical bunches of each plot (selected at random down the row) at harvest (Appendix B, Table 3b).

4.2.7 GRAPE RIPENESS MONITORING, SAMPLING AND ANALYSIS

Samples were taken weekly from pre-veraison to harvest for each experimental plot. Samples consisted of approximately 160 berries from each plot which were taken randomly. One hundred berries of the 160 berries were weighed and the volume determined by using a volumetric cylinder filled with water. Juice was pressed manually from these 160 berries. Total soluble solids (TSS), pH and total titratable acid (TA) were determined using the juice from the berries. TSS was determined with a digital refractometer (Atago Pocket PAL-1) which was zeroed with distilled water. TA was determined using an automatic titrater (Metrohm 785 DMP Tritino) with sodium (NaOH) at a dilution of 0.33 N. The pH of the juice was determined with a pH meter (Crison, Basic 20,

Lasec Laboratory and Scientific Equipment Co.). The juice mid infra red (MIR) spectrum was also scanned (Wine scan FT120 software version 2.2.1; FOSS Electric A/S, Hillerod, Denmark) for additional information on ripening parameters from calibrations for grape juice established by the chemical analytical facility at the departments of Viticulture and Oenology, Stellenbosch University, Stellenbosch, South Africa.

4.2.8 BERRY SAMPLING AND PROCESSING FOR ANALYSIS OF CAROTENOIDS, CHLOROPHYLLS AND SOME RIPENESS PARAMETERS

Fifty berries of each sub-plot at four different stages of ripeness were collected, representing pre-veraison (11 Jan 07), veraison (26 Jan 07), post-veraison (8 Feb 07) and harvest (7 Mar 07) for the 2006/2007 season. For the 2007/2008 season berries from the four ripening stages representing: pre-veraison (10 Jan 08), post-veraison (31 Jan 08), post-veraison (21 Feb 08) and harvest (3 Mar 08) were collected. These samples were collected randomly and immediately frozen in liquid nitrogen in the field to prevent breakdown and isomerisation of carotenoids and chlorophylls by enzymes, temperature and light. The frozen berries were stored at -80 ºC until processed. The seeds of the grape berries were removed while they were still frozen. Twenty-five of the 50 berries were ground to a fine powder in liquid nitrogen with an IKA A11 basic grinder (IKA®-Werke GMBH & CO.KG, Staufen, Germany) and lyophilised. The remaining 25 berries were lyophilised whole (with their seeds removed while still frozen) to give a measure of the pericarp dry weight to fresh weight ratio. This was necessary due to the incomplete recovery of homogenised tissue, and the alteration in the mass of the homogenate with the addition of liquid nitrogen, and subsequent condensation. The ground lyophilised tissue powder was stored at -80ºC and used later for chemical analyses.

4.2.9 CHEMICAL ANALYSES ON LYOPHILISED BERRY TISSUE

One hundred mg of red and fifty mg of the lyophilised grape homogenate was extracted in 50% (v/v) ethanol for one hour. Extracts were then centrifuged for 5 min at 6000 rpm. Total anthocyanin concentration of the ethanolic extract was determined using the method of Iland *et al.* (2000). Total pericarp (skin and flesh) tannin concentration was determined using the method of Sarneckis *et al.* (2006). Malic acid, glucose and fructose concentrations were determined enzymatically on ethanolic extracts decolourised with

polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) using commercial enzyme assay kits (R-Biopharm, Dramstadt, Germany).

Individual and total carotenoid and chlorophyll content of grapes berries for each sub-plot were quantified using the extraction method developed by Oliveira *et al.* (2004) with adjustments (see detailed discussion in Chapter 3). The HPLC method of Taylor *et al.* (2006) for tobacco *(Arabidopsis thaliana*) leaves was optimised for grape berries (section 3.2.6 chapter 3). Carotenoid and chlorophyll pigments were separated, identified and quantified according to the method described in Chapter 3.

4.2.10 DATA ANALYSIS

4.2.10.1 Statistical analysis

Chemical, analytical and vineyard data were analysed using Statistica 8 software. The Fisher least square test was used to indicate significant differences of mean values in one way and factorials ANOVA analysis. Scatter plots were used where data did not allow replicates, as in the case of soil water, PDWP and berry volume measurements through ripening. Comparisons of carotenoid data and ripening data between vigour and soil water content over time were analysed using factorial ANOVA. The plots originally described using the NDVI index (Section 4.2.2) were reclassified according to soil and pruning mass measurements. This classification was used to define variables in all data analyses.

4.2.10.2 Multivariate analysis

The Unscrambler software (version 9.2, CAMO ASA, Norway) was used for multivariate analysis. Principle component analysis (PCA) was used to explore vineyard data and to show possible groupings of data according to similarities in measured field variables. Partial least square analysis (PLS2) was used to explore preliminary models for predicting grape ripeness from carotenoid and chlorophyll content. For both PCA and PLS2 analysis matrixes were constructed with rows representing grape samples (objects) from experimental plots with sub-plot replicates and columns which represent chemical variables (individual carotenoids and chlorophylls). Data were pre-treated by auto-scaling in order to avoid the differences in measurement units. Auto-scaling is a widely used technique within multivariate analysis and the result is a variable with zero mean and a unit standard

deviation (Kowalski and Bender, 1972). Cross validation was used in all analysis and no outliers were removed if not specifically mentioned. The reclassified plots according to soil and pruning mass measurements were used to define variables for all data analysis (Table 4.2).

4.3 RESULTS AND DISCUSSION

4.3.1 MESOCLIMATIC DATA FOR THE VINEYARD SITE

The average day and night temperature data (from Dornier weather station) were divided according to the different block periods defined by grape ripeness stage, namely pre-veraison, veraison, post-veraison and harvest. The dates for these ripeness stages were very similar for both seasons studied. The climatic conditions for each of these ripening periods for the 2006/2007 and 2007/2008 seasons were compared (Table 4.1 a, b).

Table 4.1a Mean day and night climate differences during four ripening stages (pre-veraison; veraison; post-veraison; harvest) for the 2006/2007 and 2007/2008 ripening seasons (significant differences are only valid for each season comparing the same ripeness stage, significant differences are indicated with abcd if not bearing the same letter indicating significant difference with $p \leq 0.05$).

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Ripening stage	Date	Average day Temperature (°C)	Average night Temperature (°C)	Soil day Temperature (°C)	Soil Temperature at night (°C)	Solar Radiation ($KW/h/m^2$)	Average Sun shine per hour (%)	Rain (mm)
Pre-veraison	1-11 Jan 07	22.50ab	16.14ab	19.23a	20.16ab	1.43a	73a	0.00a
	1-11 Jan 08	22.38ab	17.23ab	19.22a	19.53b	1.36ab	68abc	0.06a
Veraison	12-25 Jan 07	25.95c	19.08c	21.54b	22.22c	1.35ab	70ab	0.00a
	12-25 Jan 08	25.12cd	17.92bc	20.12c	20.84ad	1.34ab	72a	0.01a
Post-veraison	26 Jan - 8 Feb 07	24.91ac	17.37abc	22.07b	22.37c	1.33ab	70ab	0.00a
	26 Jan - 8 Feb 08	26.06cd	17.90bc	20.75cd	21.32d	1.39a	74a	0.02a
Harvest	9 Feb - 7 Mar 07	21.47b	16.08a	20.21c	20.42a	1.17bc	60bc	0.09a
	9 Feb - 7 Mar 08	23.52ad	17.12ab	20.68d	21.04d	1.06c	58c	0.07a

Table 4.1b Mean seasonal climate differences during four ripening stages (pre-veraison; veraison; post-veraison; harvest) for the 2006/2007 and 2007/2008 seasons (significant differences are only valid for each season comparing the same ripeness stage, significant differences are indicated with abcd if not bearing the same letter indicating significant difference with $p \leq 0.05$).

Ripening stage	Date	Daily Wind Speed (km/h)	Wind Speed at night (km/h)	Daily Maximum Wind (km/h)	Maximum wind at night (km/h)	Daily relative humidity (%)	Relative humidity at night (%)
Pre-veraison	1-11 Jan 07	1.96ab	1.32ab	3.25ab	2.26abc	58.63ab	82.43ab
	1-11 Jan 08	2.13a	1.71c	3.61a	2.97d	55.30acd	72.95c
Veraison	12-25 Jan 07	1.93ab	1.23ab	3.30a	2.14ac	51.38ac	75.15c
	12-25 Jan 08	2.10a	1.50ac	3.41a	2.66bd	54.34acd	75.73c
Post-veraison	26 Jan - 8 Feb 07	2.01a	1.44ac	3.39a	2.47abd	53.90ac	79.23ac
	26 Jan - 8 Feb 08	2.08a	1.52ac	3.33a	2.59abd	47.81c	74.94c
Harvest	9 Feb - 7 Mar 07	1.91ab	1.28ab	3.21ab	2.19ac	61.39bd	81.20ab
	9 Feb - 7 Mar 08	1.68b	1.15b	2.88b	1.97c	63.56b	84.85b

In the 2007/2008 season the relative humidity pre-veraison (1-11 Jan) at night was significantly higher with significantly stronger wind movement (Table 4.1b). Significantly stronger wind was still evident at night during the veraison (12 to 25 Jan) period for this season. Significantly higher day and night soil temperatures were observed for the previous season (2006/2007) for the veraison and post-veraison stages of the ripening period. For the period classified as the harvest period (9 Feb to 7 Mar), higher average day temperature was observed in the 2007/2008 season, which was also associated with higher day and night soil temperatures than seen in the previous 2006/2007 season.

Figure 4.5 shows the average values for the vineyard mesoclimate variables per 24 hours. For the 2007/2008 season, stronger wind was more regularly observed as already been mentioned, as well as more frequent rainfall compared to the 2006/2007 season. However, more frequent decreases in solar radiation can be observed for the 2006/2007 season. Figure 4.6 shows more frequent fluctuation in daily average temperature for the 2007/2008 season than for the 2006/2007 season.

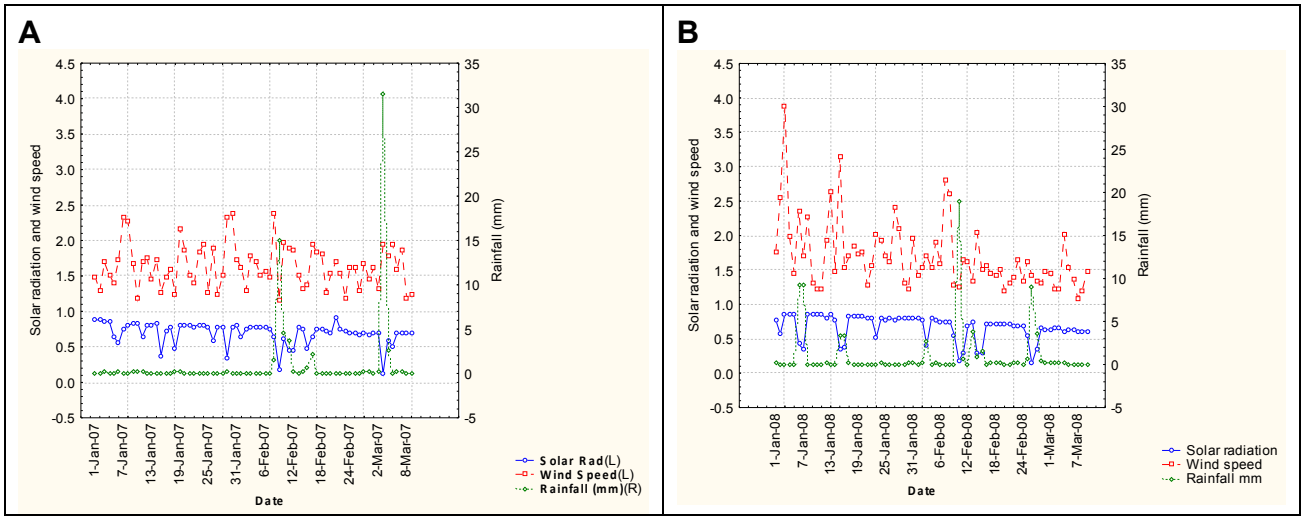


Figure 4.5 Average daily solar radiation, wind speed and rainfall (mm) (calculated as the average of 24 hours) for the A. 2006/2007 and B. 2007/2008 season respectively.

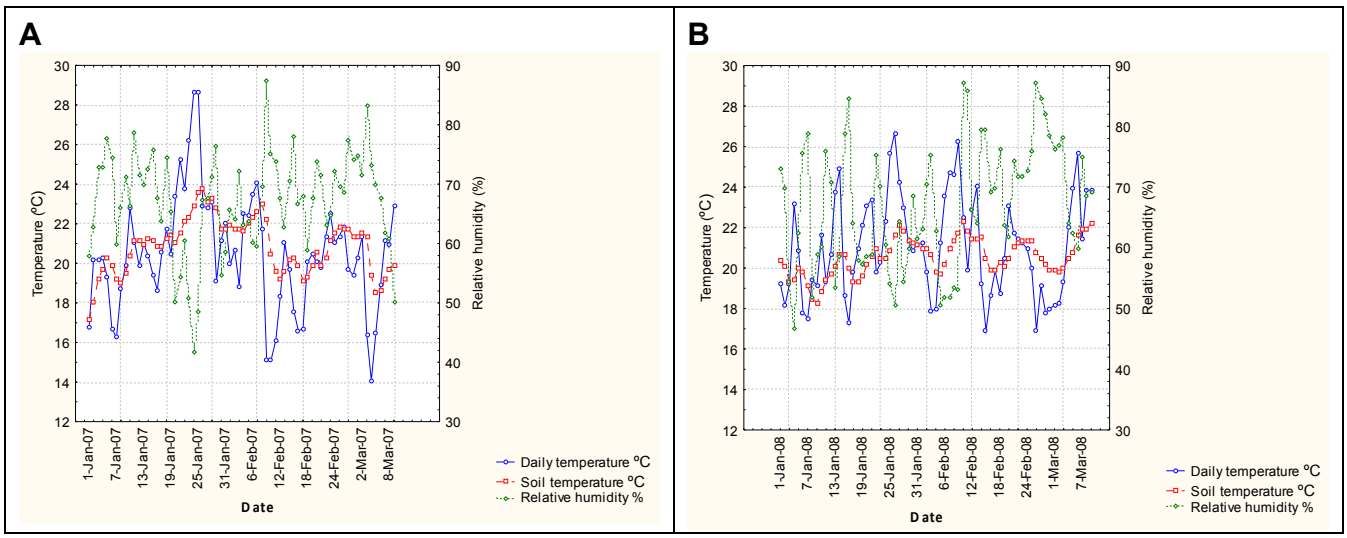


Figure 4.6 Average daily temperature, soil temperature and percentage relative humidity (calculated as the average of 24 hours) for the A. 2006/2007 and B. 2007/2008 ripening season respectively.

In conclusion, it appears that the 2006/2007 was a drier season pre-veraison since no rainfall was observed early in the season although more frequent decreases in solar radiation possibly indicates more regular cloudy weather conditions through the season with higher rainfall just before harvest compared to the latter season. The 2007/2008 season appears to be a wetter season with rainfall distributed throughout ripening but with less rainfall before harvest, which was also associated with higher temperatures.

4.3.2 PLOT DESCRIPTION

The soil water content of the different experimental plots measured with a neutron probe (Figure 4.7A and B) did not reflect the irrigation treatments applied (Appendix B, Tables 1 and 2). Lateral water movement as well as differences in the water holding capacity of the soils in different regions of the experimental site might be responsible for these conflicting results. Therefore, instead of using the original plots, the plots were reclassified according to field measurement of seasonal soil water content and pruning mass measurements to ensure greater accuracy in multivariate analyses, and discussed accordingly (Table 4.2 and Appendix B, Table 3a and 3b). Due to an error in irrigation scheduling, plot Mw 3 and Ld 1 received an unscheduled additional 44 litres water per dripper pre-veraison (11 Jan 07) in the 2006/2007 season (Appendix B, Table 1). This sudden increase in soil water can be seen in Figure 4.7A. After this additional irrigation, the soil water content of plot Mw 3

and Ld 1 decreased, and plot Ld 1 remained the plot with the lowest soil moisture content through the 2006/2007 and plot Ld 2 through the 2007/2008 season. Appendix B, Figure 1 shows the soil water content of the different experimental plots measured at 3 different depths (30 cm; 60 cm; 90 cm). From this figure it appears that maximum differences were in the 30 cm soil layer where the Ld 1 and Ld 2 plots were dryer than all the other plots throughout both ripening seasons.

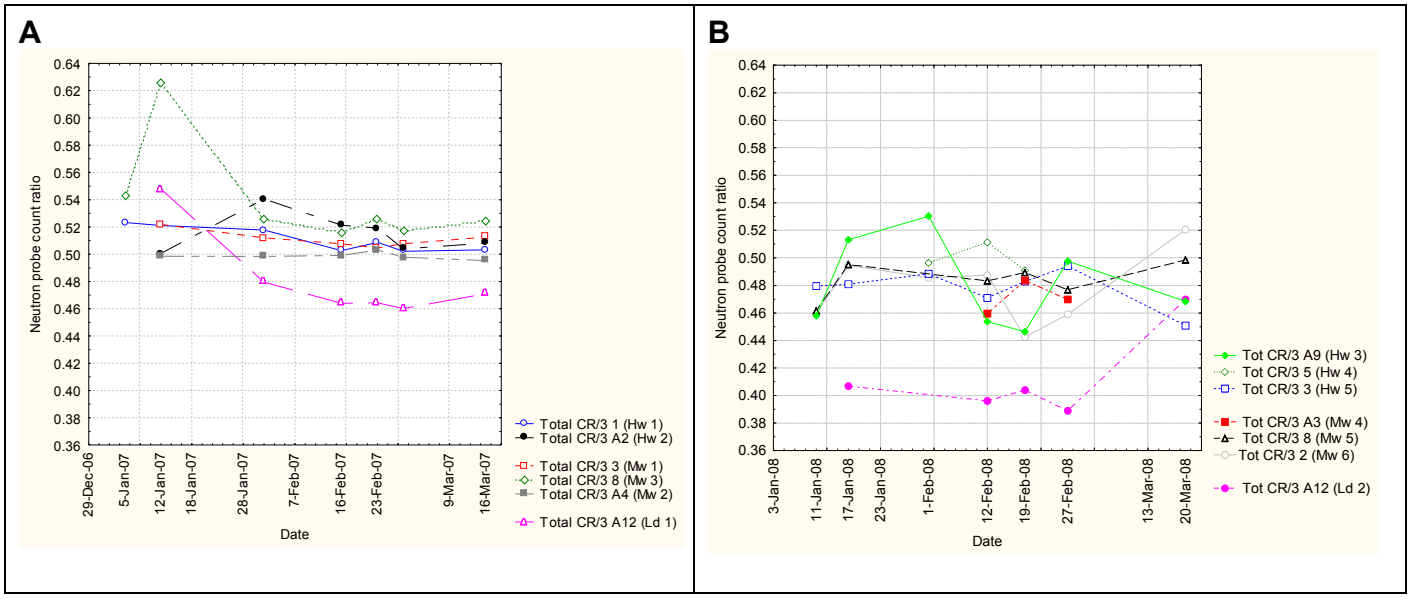


Figure 4.7 Neutron probe soil water content measurements (the sum of 3 soil depths measurements: 30, 60, 90 cm (count ratios) divided by 3 (CR/3)) for the A. 2006/2007 and B. 2007/2008 seasons respectively. Plots classified as high vigour, wet plots (Hw 1 to Hw 5); plots classified as medium vigour, wet plots (Mw 1 to Mw 6); plots classified as low vigour, dry plots (Ld 1 and Ld 2).

The PDWP of the experimental plots did not react linearly to their soil water content (Figure 4.7A, B and 4.8A, B) similar to findings by Jensen *et al*. (1998) and Carbonneau and Deloire (2001) which found that PDWP is not reduced linearly with the reduction of water availability. Ojeda *et al.* (2002) described intermediate levels of grapevine water deficit as PDWP measurements from -0.4 to -0.6 MPa.

According to this classification none of the experimental vines experienced extended periods of either severe or intermediate water stress in either season of the current study. Except for plot Mw 1 which showed intermediate stress, with PDWP measurements between 0.4 to 0.6 Mpa, on all three dates it was sampled in the 2006/2007 season (Figure 4.8A). Plot Hw 1 approached the intermediate water stress category on one of the sampling dates. For the 2007/2008 season plot Mw 6, Hw 5 and Mw 5 approached the intermediate water stress category on a post-veraison sampling date (Figure 4.8B).

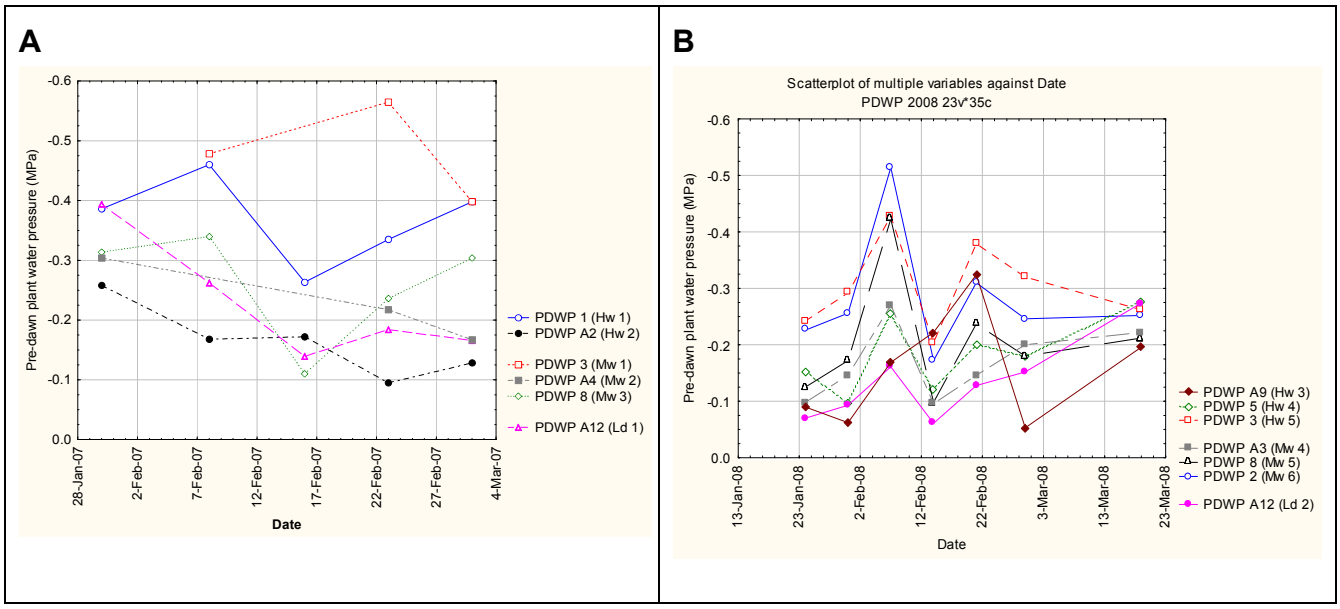


Figure 4.8 Predawn plant water potential (PDWP) for the A. 2006/2007 and B. 2007/2008 ripening seasons respectively. Plots classified as high vigour wet plots (Hw 1 to Hw 5); plots classified as medium vigour wet plots (Mw 1 to Mw 6); plots classified as low vigour dry plots (Ld 1 and Ld 2).

Table 4.2 Plot codes and description alterations.

2007 Original plot codes	2007 Original plot classification and treatments	2007 New description according to soil moisture and pruning mass measurements	2007 New plot code	2008 Original plot codes	2008 Original plot classification and treatments	2008 New description according to soil moisture and pruning mass measurements	2008 New plot code
1	High vigour dry land	High vigour wet	Hw 1	A9	High vigour irrigated	High vigour wet	Hw 3
A2	High vigour irrigated	High wet	Hw 2	5	High vigour dry land	High vigour wet	Hw 4
				3	Medium vigour dry land	High vigour wet	Hw 5
3	Medium vigour dry land	Medium vigour wet	Mw 1	A3	Medium vigour irrigated	Medium vigour wet	Mw 4
A4	Medium vigour irrigated	Medium wet	Mw 2	8	Low vigour dry land	Medium vigour wet	Mw 5
8	Low vigour dry land	Medium vigour wet	Mw 3	2	Low vigour dry land	Medium vigour wet	Mw 6
A12	Low vigour irrigated	Low vigour dry	Ld 1	A12	Low vigour irrigated	Low vigour dry	Ld 2

Multivariate analysis (PCA) was used to evaluate the variance between plots according to vineyard variables for each season. A data matrix was constructed, for PCA analysis, with experimental plots (and sub-plot samples as replicates) of the 2006/2007 season as objects and the variables: average predawn measurement of season (pdwp); average shoot diameter (~sh d); average internode length (int l); average number of shoots per vine (# sh); average number of lateral shoots (# l sh); average shoot length (sh l); average lateral shoot length (l sh); average fresh weight per berry (fw/b); average bunch mass (bu/m); yield per vine at harvest (harv); average soil water content (cr/3); average pruning mass per vine (pru/v).

The PCA analysis indicated that the model generated using the vineyard data described only 47% of the variance using two principal components (not shown). After low impact variables on the model, int l and #sh/v, were removed the model improved slightly, describing 53% of the variance using two principal components (Figure 4.9B). Essentially, the low variance in the data means that there was a high similarity between the plots in terms of the selected variables. The variables with the highest loading on PC1 were average PDWP for the season, average mass per bunch, fresh weight per berry and average yield per vine at harvest. For PC2, a higher loading on PC2 was the average number of internodes per shoot and shoot length for the season. These variables on PC1 and PC2 respectively are the variables which could potentially make the biggest contribution in separating plots (Figure 4.9A, B).

Average PDWP, bunch mass and bunch mass per vine at harvest are described by PC 1 which separates Ld 1 and Mw 1 from the rest of the plots. Ld 1 and Mw 1 had lower PDWP values, mass per bunch and bunch mass per vine at harvest. All the other plots did not clearly discriminate from each other in this regard (Appendix B, Table 3a and 3b). PC 2 is defined by number of internodes, shoot length and partially by average shoot diameter. Ld 1, Mw 2 and Mw 3 are separated by PC 2 because of their thinner, longer shoots with more internodes. This result might be explained by shoot topping on most of the remaining plots which resulted in about shorter and thicker shoots (Appendix B, Table 3a). The high vigour plots appear to correlate with higher soil water content, pruning mass per vine and pruning mass per shoot and these variables are partially described by PC1 and PC2. However, the medium vigour plots Mw 2 and Mw 3 showed larger values of lateral shoot length and number of lateral shoots.

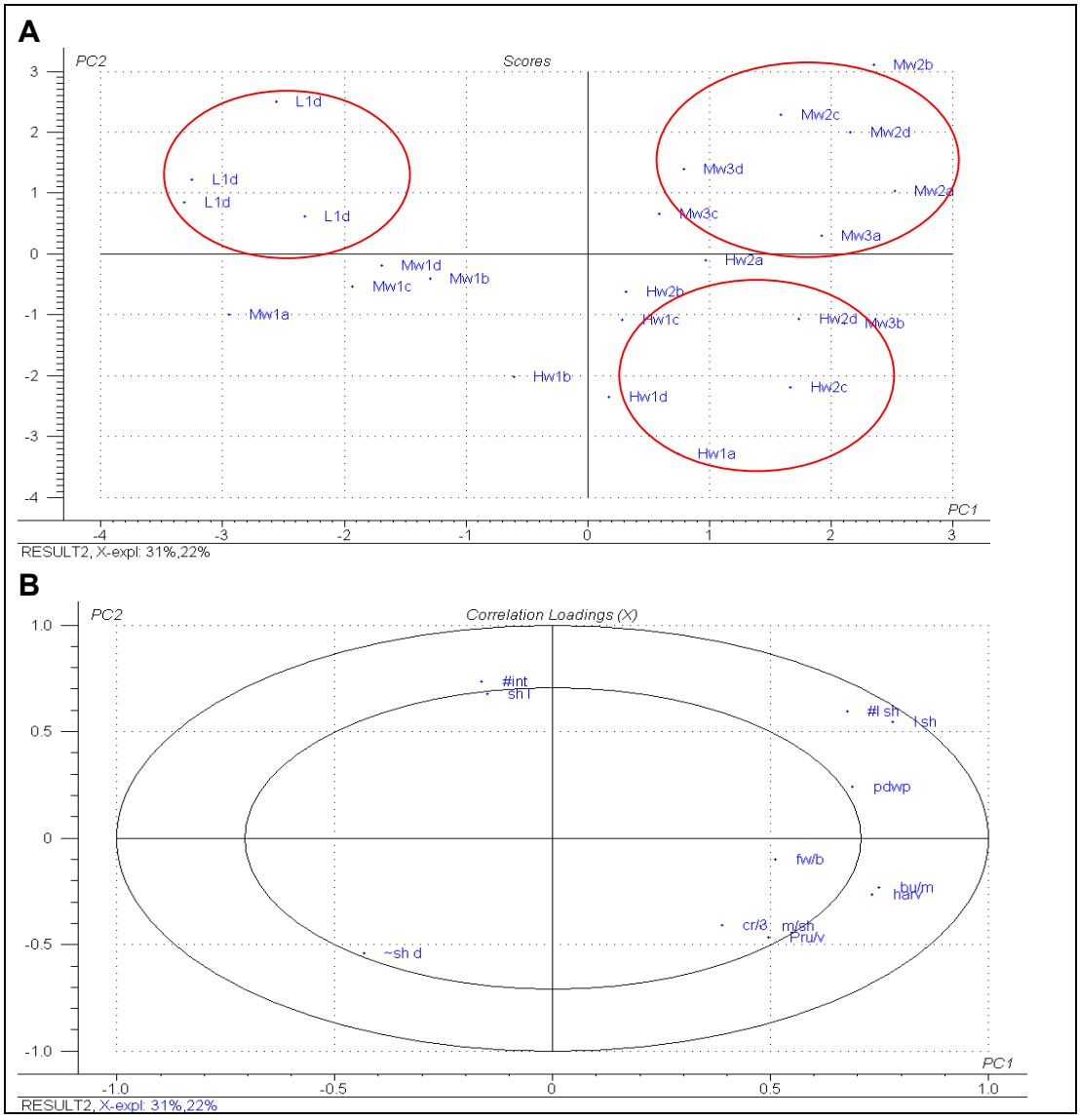


Figure 4.9 PCA analysis to discriminate between plots based on vine measurements for the 2006/2007 season. A. *Scores:* high vigour wet plots (Hw 1; Hw 2; Hw 3); medium vigour wet plots (Mw 1; Mw 2; Mw 3), low vigour plot (Ld 1), (a, b, c and d; indicate the four replicates from sub–plots). Red circles indicate grouping between samples. B. *Correlation loadings (X):* average predawn measurement of season (pdwp); average shoot diameter with pruning (~sh d); average amount of shoots per vine (# sh); average number of later shoots per shoot (# l sh); average lateral shoot length (l sh); average fresh weight per berry (fw/b); average bunch mass (bu/m); average yield per vine at harvest (harv); average soil water content (cr/3); average pruning mass per vine (pru/v average number of internodes (#int); shoot length (sh l); average mass per shoot (m/sh).

A data matrix for PCA analysis was constructed for the 2007/2008 in a similar manner to that for the previous season, but with an additional variable: average (PAR) light infiltration into the bunch zone (E/s). As with the PCA analysis of the 2006/2007 season, a poor model was generated, and the total variance described by two principal components

did not exceed 47% (model not shown). Removal of some of the poorly correlated variables with PC 1 and 2, fw/b; #bu/v; #sh and harv, improved the model, giving a total variance of 59% described (Figure 4.10B).

Higher loading weight variables on PC1 are PDWP, lateral shoot length, number of internodes, number of lateral shoots and average shoot diameter. PC1 separates plot Mw 6 and Mw 5 which has low PDWP and the least amount of lateral shoots with the shortest length (Table 3a). Higher loading weight variables on PC2 are soil water which has the highest loading weight followed by bunch mass and light measurements. On PC2, Ld 2 had the lowest soil water content and bunch mass, with all the other plots not clearly separated on PC2 (Figure 4.10B and Appendix B, Table 3a and 3b). The high vigour plots Hw 3 and Hw 4 appear to have higher values for internode length, pruning mass per vine and average shoot mass while the medium vigour plots Mw 5 and Mw 6 appears to have longer shoots.

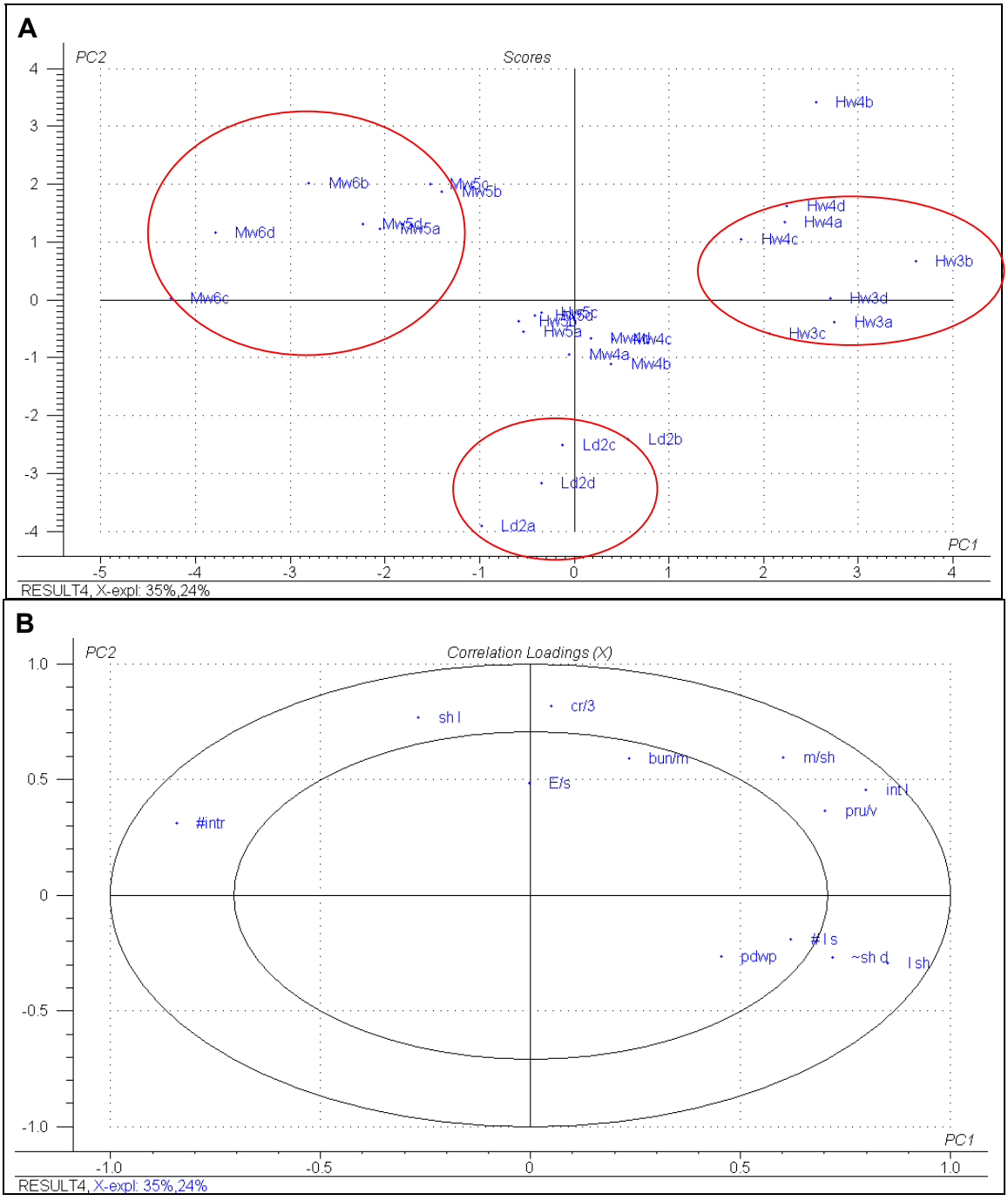


Figure 4.10 PCA analysis to discriminate between plots based on vine measurements for the 2007/2008 season. A. *Scores:* high vigour, wet plots (Hw 3; Hw 4; Hw 5); Medium vigour, wet plots (Mw 4; Mw 5; Mw 6) and the low, vigour plot (Ld 2), (a; b; c and d; indicates the four replicates from sub–plots). Red circles indicate grouping between samples. B. *Correlation loadings (X):* Average predawn measurement of season (pdwp); average shoot diameter (~sh d); average internode length (int l); average number of internodes per shoot (#intr); average number of later shoots per shoot (# l s); average bunch mass (bun/m); average yield per vine at harvest (harv); average soil water content (cr/3); average pruning mass per vine (pru/v); average shoot mass (sh

m); average shoot length (sh l); average (PAR) light infiltration into the bunch zone (E/s); average lateral shoot length (l sh)

For both seasons significant differences in vigour were evident driven by vigour measurements such as pruning mass per vine and mass per shoot. The 2006/2007 season yield component also made a contribution in separating the different vigour plots. Soil water content appears to correlate with pruning mass and mass per shoot while PDWP was more closely correlated with lateral shoot growth in 2006/2007.

In 2007/2008, there was little variation in yield components found across the vineyard. Since there was higher rainfall early in the period of rapid shoot growth, pre-veraison, this most likely drove increases in vigour across the whole site. However, this series of rainfall events occurred post-set, and may therefore explain the poor correlation of vigour with yield components. Harvest was down in 2007/2008 compared with the previous season (Appendix B, Table 3b), but the distribution of this measure between plots was smaller than in 2006/2007, bringing about the poor correlation of yield components with measures of vigour. In other words, for the 2007/2008 season, vigour differences were evident, but did not drive yield components as strongly as 2006/2007. It appears from the PCA analysis that the average seasonal soil water content did play a significant role in both seasons as a driver of the yield variables. In the 2006/2007 season average yield per vine contributed to separating experimental plots but was removed from the 2007/2008 model due to it being an insignificant variable in the original PCA analysis. Thus, it appears that the soil water content did not drive yield as strongly as in 2006/2007, but mainly limited lateral shoot growth in some of the plots later in the season. This is most likely because water was not limiting during the period of rapid shoot growth earlier in the season but became limiting during the period of lateral shoot growth. In the 2007/2008 season, PDWP is not strongly correlated with soil water content on the PCs, but is partially correlated with it, negatively, which is not expected. In both seasons PDWP was positively associated with lateral shoot length. This indicates that the strongest impact of changes in PDWP in grapevines was on lateral shoot growth. More lateral shoot growth was associated with higher PDWP (less stress). This is an expected result, since in grapevines the most sensitive indicator to plant water status is lateral shoot growth.

However, as discussed previously, none of the plots were in either intermediate or severe water stress by Ojeda *et al*. 's (2002) definition, which explains the poor correlation of PDWP and either vigour components or yield components for either season.

4.3.2.1 Descriptive comparison of two extreme plots

When the two extreme plots in regard to the measurement of soil water content and grapevine vigour variables were compared for the two seasons of the study plot Ld 1 had a significantly lower seasonal (2006/2007) average in soil water content of 0.48 (neutron probe count ratio) compared to plot Hw 1 with a seasonal soil water content of 0.52 (neutron probe count ratio). For the 2007/2008 ripening season significantly lower seasonal soil water content (0.41) was observed for plot Ld 2 compared to plot Hw 3 with soil water content of 0.51 (Figure 4.7 and Appendix B, Table 3b). The difference in soil water content between these plots was more apparent when the soil water content for the three soil depth measures was compared at different time points in the season (Figure 4.11A), such that in 2006/2007, Ld 1 was drier than Hw 1 primarily at soil depth 30 cm. In 2007/2008, Ld 2 was drier than Hw 3 at both the 30 cm and 60 cm soil depths. Smaller differences in soil water content at a depth of 90 cm were observed when low and high vigour plots were compared for both seasons. From Figure 4.11B it appears that water in the deeper layers of the dry plot was not lacking throughout the ripening period. The grapevine's root system may have allowed the uptake of this water from this layer which could potentially explain the lack of significant difference in predawn leaf water potential between high vigour wet and low vigour dry plots.

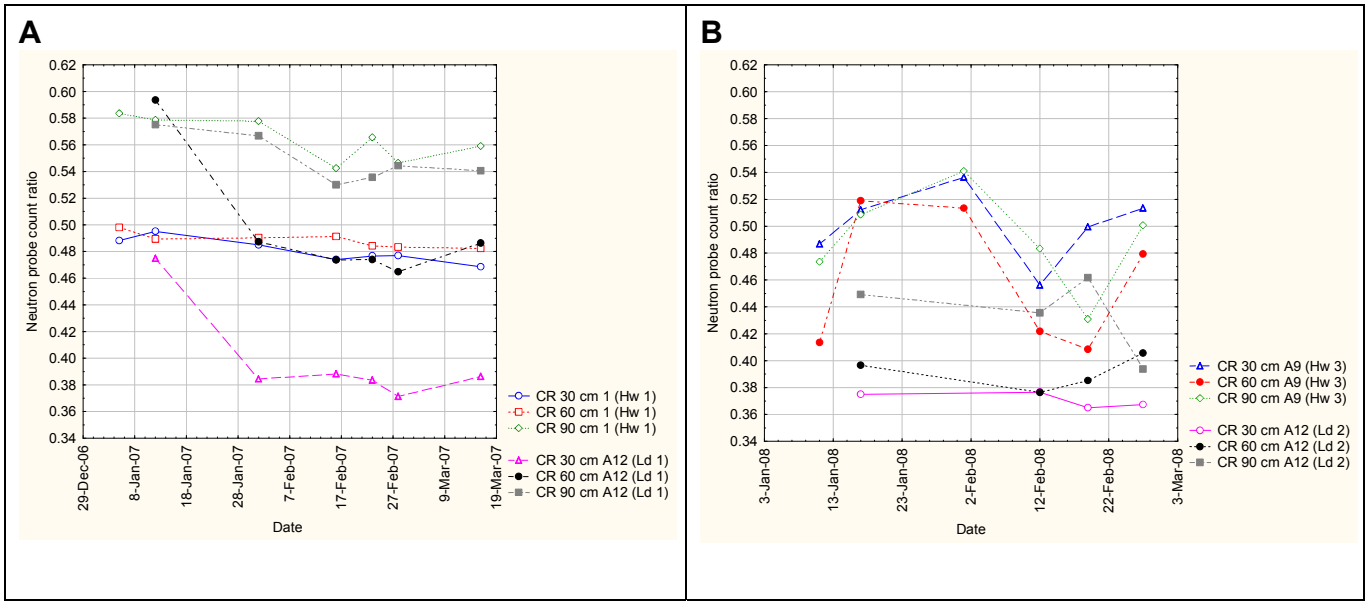


Figure 4.11 Neutron probe count ratio's of high vigour wet (Hw 1 and Hw 3) and low vigour dry (Ld 1 and Ld 2) plots on 3 selective depths (30 cm; 60 cm; 90 cm) for A. the 2006/2007 and B. 2007/2008 respectively.

It is important to note that plots Ld 1, Ld 2 and Hw 3 were irrigated (Appendix B, Table 1 and 2) and plot Hw 1 was minimally irrigated. One would thus expect that there would be higher soil water content in the soils from these plots which are not the case here. The PDWP of the selected plots did not react linearly to their soil water content (Figure 4.11A and B) as discussed in Section 4.3.2. According to the classification of Ojeda *et al.* (2002), neither the high vigour, wet vines (Hw 1; Hw 3) nor the low vigour, dry vines (Ld 1; Ld 2) experienced extended periods of either severe or intermediate water stress in either season of the current study, but the Hw 1 plot approached the intermediate water stress category on two of the sampling dates (Figure 4.12A). Differences in root distribution for the selected plots might have played a role in the availability of water to the plant and also affected the response of the grapevine to environmental conditions.

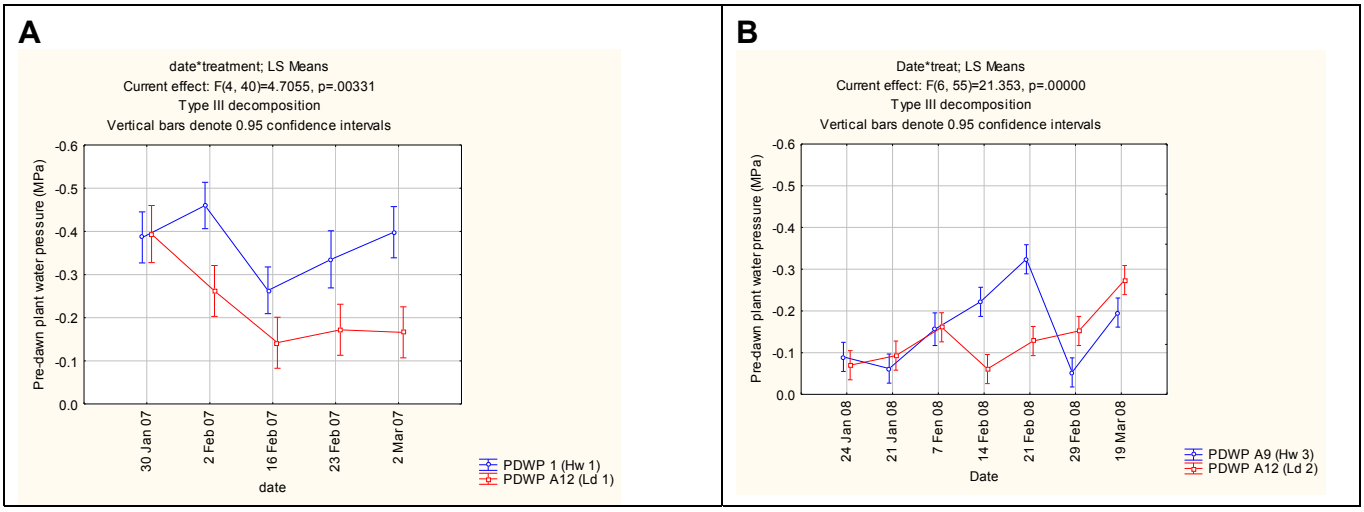


Figure 4.12 Predawn plant water pressure (PDWP) of selected high vigour wet (Hw 1 and Hw 3) and low vigour dry plots (Ld 1 and Ld 2) for the A. 2006/2007 and B. 2007/2008 season respectively.

Plot Ld 1 had a pruning mass of 0.63 kg per vine which is significantly lower than the high vigour plot Hw 1 with a pruning mass of 1.01 kg in the 2006/2007 season. In the 2007/2008 season the low vigour plot Ld 2 had a pruning mass of 0.95 kg per vine compared to the significantly higher pruning mass of the high vigour plot Hw 3 of 1.31 kg per vine. Plots Ld 1 and Hw 1 in the 2006/2007 season and Ld 2 and Hw 3 in the 2007/2008 season showed significant differences in vigour and total soil water content throughout the ripening period. Therefore, these plots were selected to explore the influence of vigour differences and soil water content on pigment development and other ripening parameters in berries of two extremes: high vigour, wet and low vigour, dry plots. Differences in grapevine water status were absent as indicated by changes in PDWP.

4.3.2.2 Descriptive comparison of ripening parameters in two extreme plots

The weight, volume and soluble solids of grape berries from the selected extreme plots are shown in figure 4.13. It is evident from figure 4.13a that the total soluble solids were decreased “diluted” only at one sample stage post-veraison (7 to 15 February 07) for plot Hw 1. This increase in berry size correlated with a large amount of rain more than 15 mm for the first time in the season and little irrigation before hand (Appendix B, Table 1). However, berry weight did not linearly correlate with the berry volume for the low vigour plot for one sample post veraison just before harvest in the 2006/2007 season which

indicates that separation in the berry weight:volume ratio occurred (Figure 4.13A). Non linearity was also observed between the berry weight and volume for a sample date post-veraison for the high vigour plot in the 2007/2008 season. It appears that the amount of water (volume) in the berry especially post-veraison drives the weight of the berry more than the amount of soluble solids present in berries. In the 2007/2008 season the berry weight tended to decrease while the soluble solids were still increasing due to a decrease in volume/water.

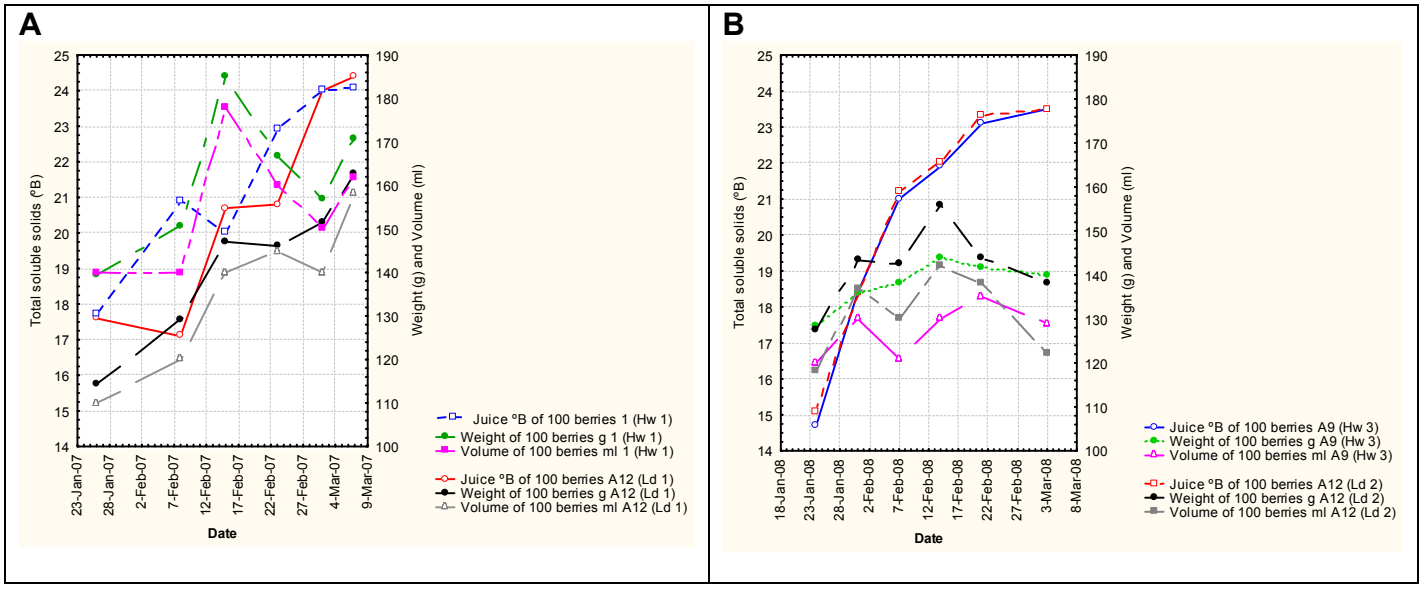


Figure 4.13 Juice total soluble solids, weight and volume of 100 berries between the high vigour, wet plots (Hw 1; Hw 3) and low vigour dry plots (Ld 1; Ld 2) for the A. 2006/2007 and B. 2007/2008 ripening seasons respectively.

The malic acid, total glucose and fructose, total tannin and anthocyanin content of the selected high and low vigour plots for each season was investigated on a per berry fresh weight (fw) and mg/g fw basis in order to observe differences in loading/synthesis or degradation/conversion of these compounds (Appendix B, Figure 2 and 3).

For the 2006/2007 season malic acid per berry fresh weight was significantly higher in berries of Hw 1 than in Ld 1 pre-veraison (11 Jan 07) (Appendix B, Figure 2A). However, from veraison to harvest no significant difference in malic acid content was observed for either of the seasons studied between the low dry and wet high vigour plots (Appendix B, Figure 2A and 3A). Hawker (1969) states that malic acid, is metabolized as an energy source during the second growth phase. In the current study it appears that the rate of metabolizing of malic acid was not altered by different vigour vines.

For both seasons the high vigour wet plots showed a significantly higher per berry fw content of total glucose and fructose post-veraison (8 Feb 07, 21 Feb 08) compared to the low vigour dry plots although no difference between berries were observed at harvest. However, for the 2006/2007 season, berries of Hw 1 reached their maximum total glucose and fructose concentration immediately post-veraison while the total glucose and fructose concentration per berry for berries from plot Ld 1 continued to increase until harvest (Appendix B, Figure 2B). For both seasons the high vigour wet plot berries reached their final total glucose and fructose concentration earlier than the low vigour plots, and then stabilised (Appendix B, Figure 4B). Although, in the 2007/2008 season, unlike the 2006/2007 season, berries from the Ld 2 plot stabilised at a maximum in total hexose sugars post-veraison (31 Jan 08). Wang *et al.* (2003) found that sugar unloading in berries is inhibited in ripening berries during water deficiency stress. But as already discussed in this section, water was not limiting in these grapevines. Rather, it is possible that a reduction in the leaf area:crop load ratio caused a delay in sugar accumulation in the lower vigour vines (Bindon 2008 a, b).

Total tannin per berry fw was significantly lower in plot Ld 1 post-veraison (8 Feb 07) compared to plot Hw 1 (Appendix B, Figure 2C). The total pericarp tannin content in the berries of both plots decreased post-veraison with no significant differences observed at harvest. Total tannin was lower post-veraison (31 Jan 08), in the berries of plot Ld 2, but increased to significantly higher concentration (mg/berry fw) post-veraison (21 Feb 08) compared to the berries of the high vigour wet plot (Hw 3) (Appendix B, Figure 3C). It seems that the increase of tannin for the berries of the Ld 2 plot was triggered later (from 31 Jan 08 to 21 Feb 08) although to higher levels, compared to the Hw 3 plot. After 21 Feb 08, both plots showed decreases in extractable tannin towards harvest with no differences evident by harvest (Appendix B, Figure 3C). Since this study is one of few which have reported viticultural data using the methyl cellulose precipitate (MCP) method of Sarneckis *et al.* (2006), the increase in tannin post-veraison is difficult to interpret in the light of other studies. Randomisation of the samples during analysis meant that differences in extraction conditions or the method itself would have been detected, and as such, the observed increase in MCP tannin was accurate.

The total anthocyanin concentration per berry fw from plot Ld 1 increased significantly from non detected pre-veraison (11 Jan 07) to harvest (7 Mar 07) while berries from plot Hw 1 only showed significant increases until shortly post-veraison (8 Feb 07) (Appendix B,

Figure 2D). However, no significant differences in total anthocyanin content per berry fw were observed between plots Ld 1 and Hw 1 through ripening. Comparing concentrations of berries from the high vigour plot and low vigour plot no significant differences on a mg/g basis for any of the respective ripening stages were observed in either the 2006/2007 or 2007/2008 season. These results can be explained by the PDWP data which indicated that none of the experimental vines experienced water stress. Thus the soil water effect did not transfer through to give alterations in berry weight that were highly significant.

From the results in this descriptive comparison of two extreme plots representing differences in grapevine vigour it is evident that the vigour and the soil water conditions in this study did not significantly alter ripeness parameters on a per berry fw basis at harvest. The most apparent alteration between the plots was the rate of sugar loading measured as hexoses per berry.

4.3.3 EFFECT OF VIGOUR AND SOIL WATER CONTENT ON THE CAROTENOID AND CHLOROPHYLL CONTENT OF GRAPES

Based on the plot description and comparisons detailed in section 4.3.2, a guideline for the interpretation of pigment profile analytical data in grapes was generated. For this section of the chapter, a general PCA analysis of grape pigments at different ripeness stages and from different plots will be discussed. Based on the results of section 4.3.2, a poor separation in plot characteristics was achieved using PCA analysis. As a result of this limitation in the study, a descriptive comparison of the pigment analysis of the two extreme plots will also be discussed.

4.3.3.1 PCA analysis of pigment profiles in grapes from all plots

PCA analysis was conducted to evaluate differences, if any, in the carotenoid pigment profile, chlorophylls and other ripening parameters expressed as content per berry fw. In other words, PCA analysis was used to evaluate any clustering of ripening parameters (data not shown), carotenoid and chlorophyll data according to variation in the experimental plots.

A data matrix was constructed for PCA analysis with grape samples of each plot as objects and the individual carotenoids and chlorophylls in µg per berry fresh weight at different ripening stages of the 2006/2007 season (pre-veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) as variables.

High loading weights for the variables on PC1 are all the individual carotenoids and chlorophylls (total chlorophyll and chlorophyll a) except for the carotenoid 5,8-epoxy-β-carotene and chlorophyll b (Figure 4.14B and Appendix B, Table 5). PC2 describes 5,8-epoxy-β-carotene as high loading variable. The model describes 78% of the total variance in the data when two PCs are used. The PCA analysis indicates a high level of correlation between all pigments with the exception of 5,8-epoxy-β-carotene. The results indicate that the strongest driver within the data was changes in the variables following the progression in ripening with no clustering of data according to experimental plots. The observed trend was higher amounts of chlorophyll a, β-carotene and *cis*-β-carotene pre-veraison, all of which decreased with increasing ripeness. Conversely, the carotenoid 5,8-epoxy-β-carotene had higher concentrations per berry in later ripening stages with the highest content post-veraison 8 Feb 07 (Figure 4.14).

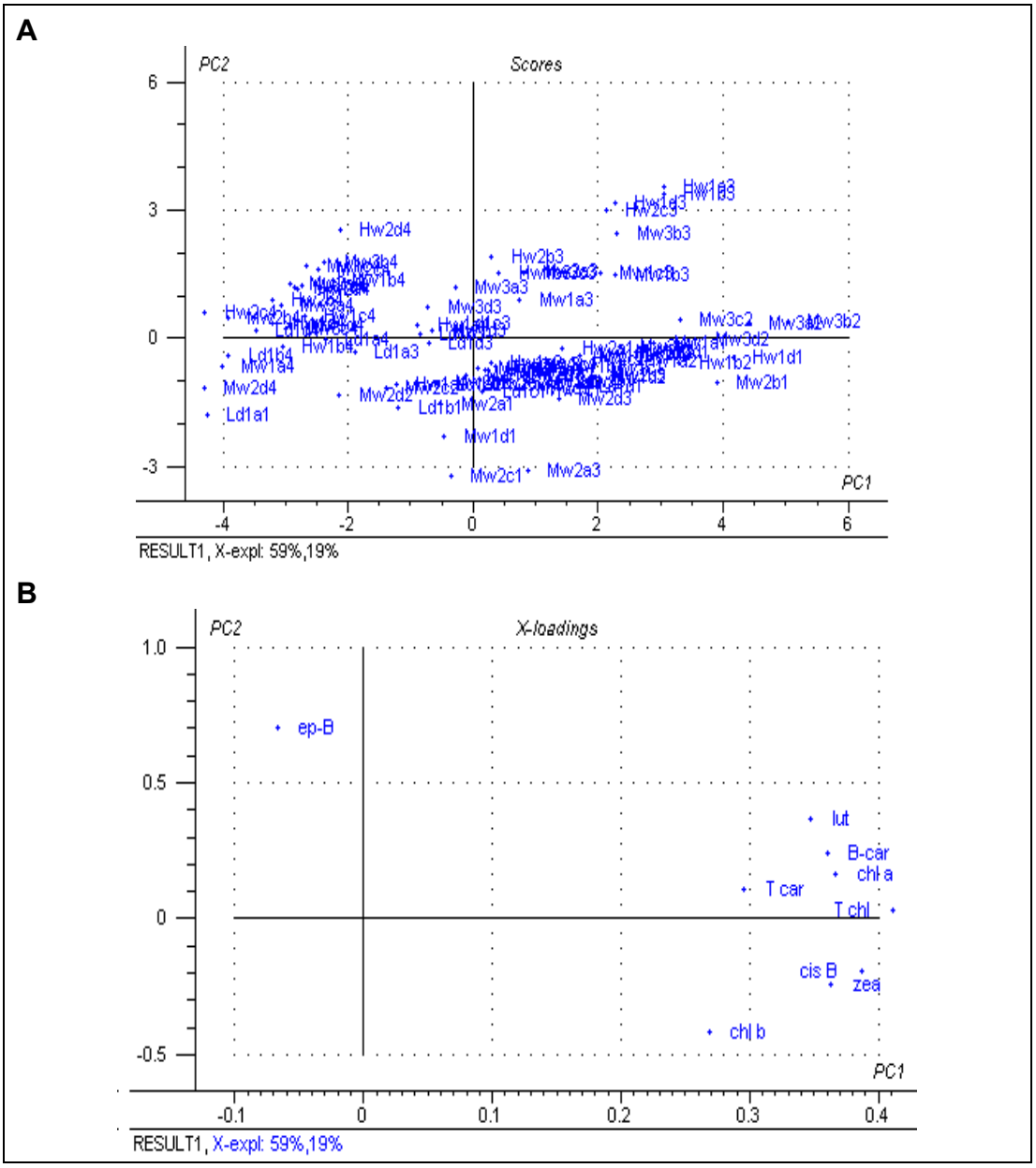
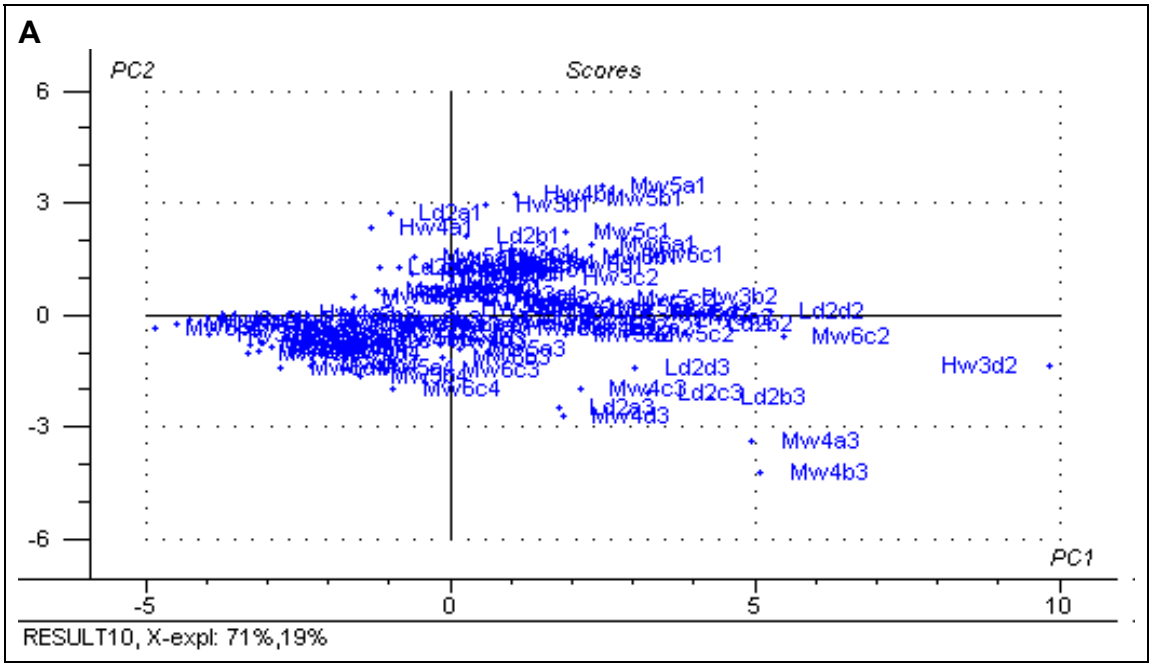


Figure 4.14 PCA analyses of carotenoid and chlorophyll per µg/berry fresh weight. A. Scores*:* high vigour wet plots (Hw 1;Hw 2); medium vigour wet plots (Mw 1; Mw 2; Mw 3) and low vigour plot (Ld 1), (a; b; c and d; indicates the four replicates from sub-plots) of four ripening stages during the 2006/2007 season: pre-veraison 11 Jan 07 (1); veraison 26 Jan 07 (2); post-veraison 8 Feb 07) (3); harvest 7 Mar 07 (4). B. *X-loadings:* 5,8-epoxy-β-carotene (ep-B); lutein (lut); β-carotene (B-car); chlorophyll a (chl a); *cis*- β-carotene (cis-B); zeaxanthin (zea); chlorophyll b (chl b); Total carotenoids (T car); Total chlorophyll (T chl).

The same multivariate analysis was conducted on the 2007/2008 season ripening parameters (data not shown), carotenoid and chlorophyll data (Figure 4.14).

Similar results were obtained as for the 2006/2007 season with the high loading variables on PC1 being all the individual carotenoids and chlorophylls except 5,8-epoxy-β-carotene and differently to the 2006/2007 season, zeaxanthin. PC2 describes 5,8-epoxy-β-carotene, and zeaxanthin as loading variables. The PCA analysis shows the progression in ripening with higher amounts of most of the carotenoids and chlorophylls pre-veraison and decreases in these components with ripening. Again, no clustering was observed according to experimental plots. Zeaxanthin had higher concentrations earlier in the ripening season, while 5,8-epoxy-β-carotene, had higher concentration per berry in the later stages of ripening, with the highest content post-veraison (21 Feb 08) (Figure 4.14 and Appendix B, Table 6.



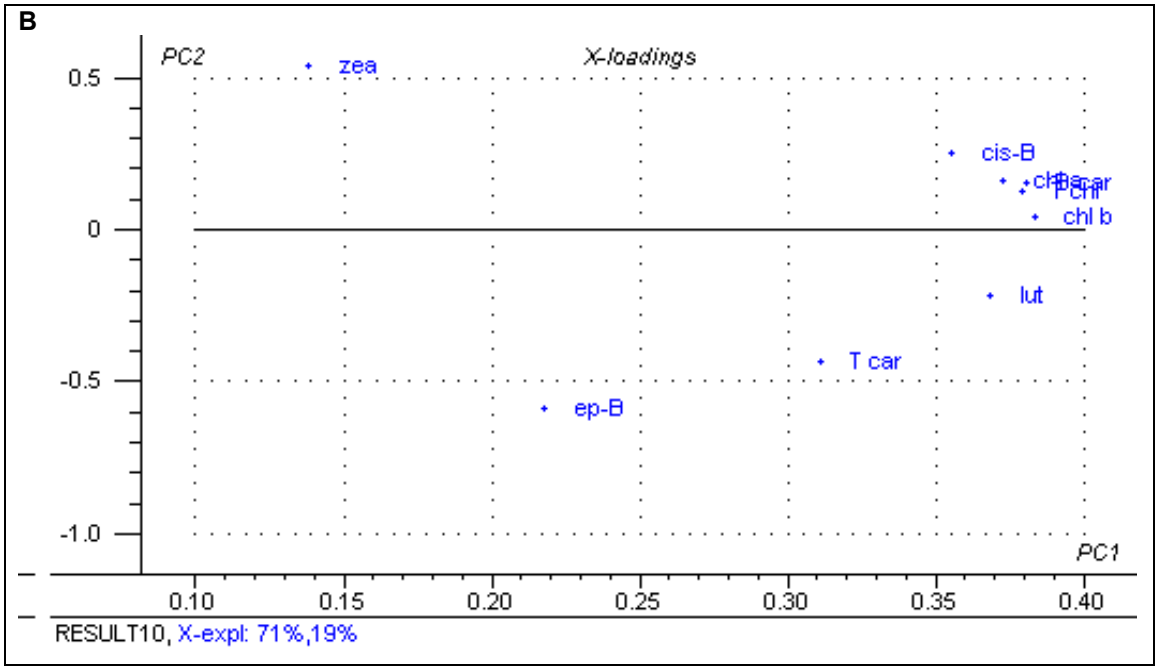


Figure 4.15 PCA analysis of carotenoid and chlorophyll per µg/berry fresh weight. A. *Scores:* high vigour wet plots (Hw 3; Hw 4;Hw 5); medium vigour wet plots (Mw 4; Mw 5; Mw 6) and low vigour plot (Ld 2), (a; b; c and d; indicates the four replicates from sub-plots) of four ripening stages during the 2007/2008 season: pre-veraison 10 Jan 08 (1); post-veraison 31 Jan 08 (2);post-veraison 21 Feb 08 (3); harvest 3 Mar 08 (4). B. *X-loadings:* 5,8-epoxy-β-carotene (ep-B); lutein (lut); β-carotene (B-car); chlorophyll a (chl a); *cis*- β-carotene (cis-B); zeaxanthin (zea); chlorophyll b (chl b).

For both seasons of the study, no clustering of data according to vigour measures and water content were observed (Figure 4.13 and 4.14). However, in both, different ripening stages could be discriminated by PCA analysis. This result is expected, based on the limited variability observed in the vineyard parameters studied. As discussed previously, no water stress was experienced by the vines according to the definition of Ojeda (2002). Where large differences in carotenoid concentration in response to variable conditions in soil and grapevine water status have been observed, water stress was caused by low water retention soils which resulted in an increase in carotenoid content for all the carotenoids analysed: lutein, β-carotene, neoxanthin, violaxanthin and luteoxanthin (Oliveira *et al.* 2003).

However, in that same study on high water-retention capacity soil, there was no effect on carotenoid content comparing irrigated and non-irrigated treatments. Oliveira *et al.* (2003) showed that the response of the carotenoids to water stress occurred in fruit from

an early stage of development, and the effect on carotenoid content was retained as the fruit matured.

4.3.3.2 Descriptive comparison of pigment profiles during ripening in grapes from two extreme plots

Selected high, wet and low vigour, dry plots for each season were further investigated to evaluate the effect of vigour and soil water content on the profile of individual carotenoids as well as the total carotenoid and chlorophyll content of berries. From graphs obtained by comparing selected high vigour wet and low vigour dry plots it was evident that maximum differences were most apparent at the post-veraison sampling date 8 Feb for the 2006/2007 season and 21 Feb for the 2007/2008 season (Appendix B, Figures 4 to 7). Multivariate analysis was conducted on these specific sampling dates respectively combined with the vineyard variables which significantly separated the experimental plots described in Section 4.3.2 (Figure 4.9 and 4.10).

Maximum differences were seen post-veraison for both seasons although the dates differed for the two seasons studied. For the 2006/2007 season higher pigment contents shortly post-veraison were associated with higher vigour plots (Appendix B, Figure 8A and 8B). For the 2007/2008 season the converse, higher contents of carotenoids were found in the lower vigour plots (Appendix B, Figure 9A and B). However, in each season, there were no clear association of vineyard parameters evident. In 2006/2007, the higher pigment content appeared to be driven by higher values in yield components, higher total glucose and fructose and higher soil water content but was not strongly associated with vigour measures. In 2007/2008, the converse happened, with the increase in pigments post-veraison associated with lower vigour vines, and was positively associated with lateral shoot growth but not associated with yield components (Appendix B, Figure 9A and B). In this season, higher pigment contents post-veraison was negatively correlated with PDWP and soil water content.

In conclusion, from the vineyard analysis, the plots separated according to different parameters for each vintage, and the net responses in terms of growth (vigour) and yield components were not consistent due to seasonal differences. There was a limited effect if any of vineyard variability on the pigment profiles, although shortly post-veraison some

differences could be observed, but these appeared to be associated with the timing of carotenoid and chlorophyll degradation since levels were similar by harvest.

4.3.3.3 Changes in ripening parameters carotenoid and chlorophyll content during ripening

From the PCA analysis shown in section 4.3.3.1 it is evident that the ripeness stage was responsible for driving most of the variation in the data, describing more than 78% of the variation in the data. Significant differences at harvest between the two ripening seasons are shown in Table 4.3.

Table 4.3 Significant differences of individual and total carotenoids, chlorophylls and ripening parameters at harvest for the 2006/2007 and 2007/2008 seasons.

Date	Maturation stage	Zea	Lut	ep-β-car	β-car	*Cis* β-car	Total Car	Chl a	Chl b	Total Chl	Malic acid	Tot (Glucose + Fructose)	Total Tannin	Total Anthocyanin
**Average ug/berry fresh weight											**Average mg/berry fresh weight			
7-Mar-07	harvest	0.01a	1.55a	0.28a	0.62a	0.05a	2.61a	11.74a	2.50a	16.13a	0.85a	148.77a	0.95a	21.15a
3-Mar-08	harvest	0.03b	1.72b	1.55b	0.62a	0.08b	4.49b	9.02b	3.84b	13.26b	2.74b	146.66a	0.70b	18.42a

**Average (Ave) calculated from all experimental plots including their four biological replicates and three analytical replicates for each season respectively.
Carotenoids: Zeaxanthin (Zea); Lutein (Lut); 5,8-epoxy-β-carotene (ep-β-car); β-carotene (β-car); *cis* β-carotene (*cis* β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds.
Chlorophylls: Chlorophyll a (Chl a); Chlorophyll b (Chl b); Total chlorophyll (Tot chl) calculated as the sum of all chlorophylls and detected chlorophyll derivatives.
Significant differences: indicated with ab when not bearing the same letter indicating significant difference with $p \leq 0.05$ between seasons.

Individual ripening parameters, carotenoids and chlorophylls determined on a per berry basis were investigated for both ripening seasons studied, with reference to the possible effect of climatic variation between seasons. It is important to take note that the dates indicated on the graphs Figure 4.16 - 4.19 do not represent the same ripening stages for both seasons.

Malic acid decreased significantly from pre-veraison to harvest on a per berry basis in both seasons (2006/2007 and 2007/2008) (Figure 4.16). Hawker (1969) stated that malic acid, is metabolized as an energy source during the second growth phase. A significant difference at harvest were observed when the two seasons were compared with 0.85 mg/berry malic acid for the 2006/2007 season compared to the 2.74 mg/berry malic acid at harvest for the berries of the 2007/2008 season (Table 4.3). Ruffner *et al*. (1976) reported

that temperature is the main factor determining the malate concentration in mature berries. In this study significantly higher values of malic acid mg per berry were observed for the 2007/2008 season which had higher temperature during the period classified as harvest (Table 4.1).

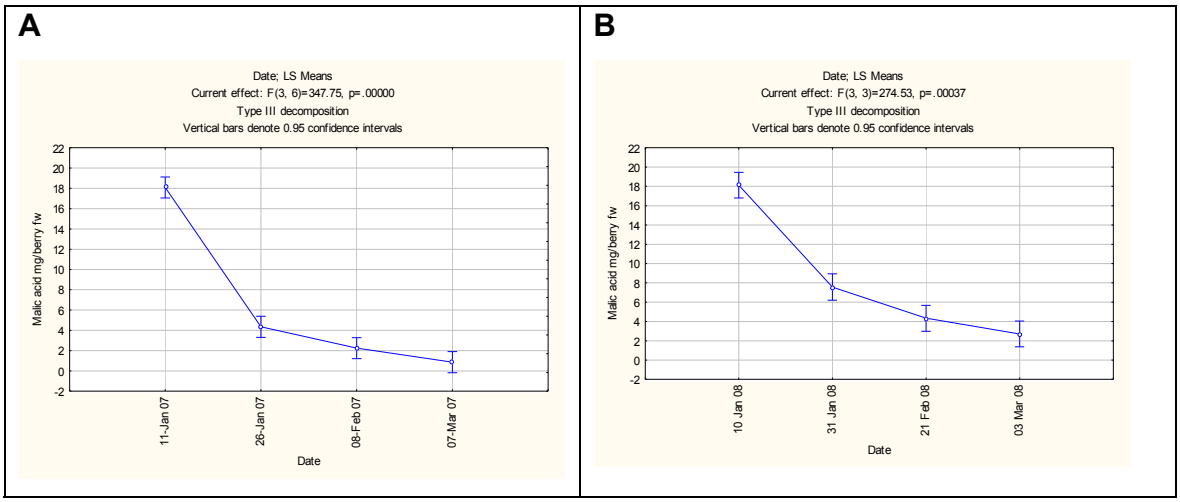


Figure 4.16 Malic acid content per berry fw at four ripening stages during the A.2006/2007 (pre-veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) and B. 2007/2008 (pre-veraison 10 Jan 08; post- veraison 31 Jan 08; post-veraison 21 Feb 08; harvest 3 Mar 08) seasons respectively.

Total hexose sugars, calculated as the sum of glucose and fructose per berry increased significantly from pre-veraison to harvest with no significant difference between the total glucose and fructose mg/berry fw between the two seasons at harvest (Table 4.3 and Figure 4.17). Higher variability in this measure was seen in the 2007/2008 season which might be due to the greater number of experimental plots included in the study in this season.

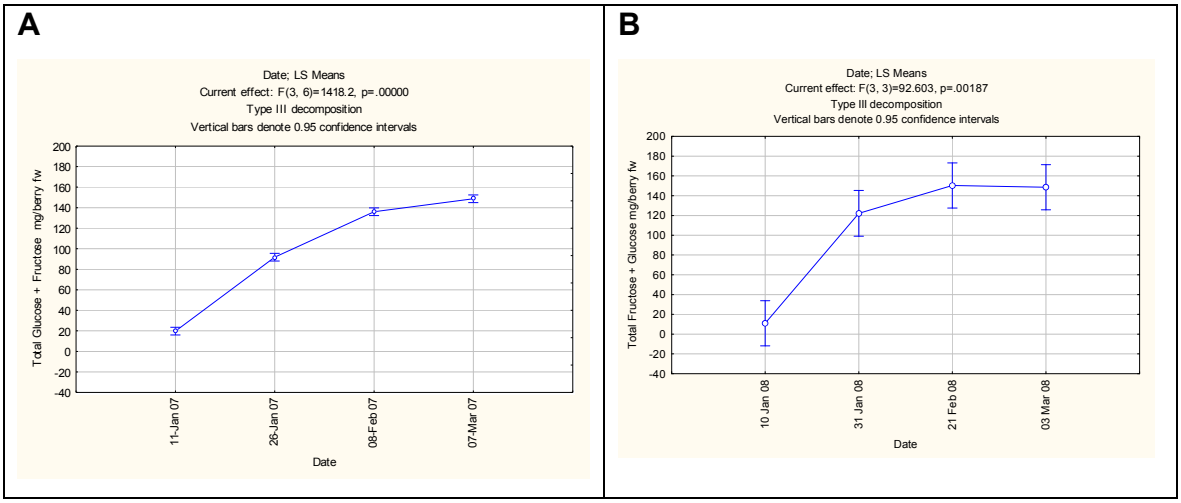


Figure 4.17 Total glucose and fructose content per berry fw at four ripening stages during the A. 2006/2007 (pre-veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) and B. 2007/2008 (pre-veraison 10 Jan 08; post- veraison 31 Jan 08; post-veraison 21 Feb 08; harvest 3 Mar 08) seasons respectively.

The tannin content measured for the 2006/2007 season showed a significant increase from veraison to post-veraison per berry fresh weight and a significant decrease to harvest (Figure 4.18). Downey et al. (2003) also found a decrease in the tannin levels in Shiraz berries from veraison to harvest. No significant differences were observed through the ripening season per berry for the 2007/2008 season. It must be noted that there was a much higher biological variability in the tannin values determined for the plots of 2007/2008 compared to 2006/2007 which could have obscured changes during ripening (Figure 4.18). However, these results agree with Habertson *et al*. (2002) who reported that tannins in the hypodermal tissue are synthesised very early in berry development and change very little from veraison to harvest on a per berry basis. A significant difference of total tannin at harvest was observed between the two seasons with 0.95 mg/berry fw for the 2006/2007 season compared to the 0.70 mg/berry fw of the 2007/2008 season. Downey *et al*. (2003) also found a significant seasonal influence on the tannin levels of grape berries. As discussed in section 4.3.2.2 this study is one of few which have reported viticultural data using the methyl cellulose precipitate (MCP) method of Sarneckis *et al.* (2006), the increase in tannin post-veraison is difficult to interpret in the light of other studies. Randomisation of the samples during analysis meant that differences in extraction conditions or the method itself would have been detected, and as such, the observed increase in MCP tannin was accurate.

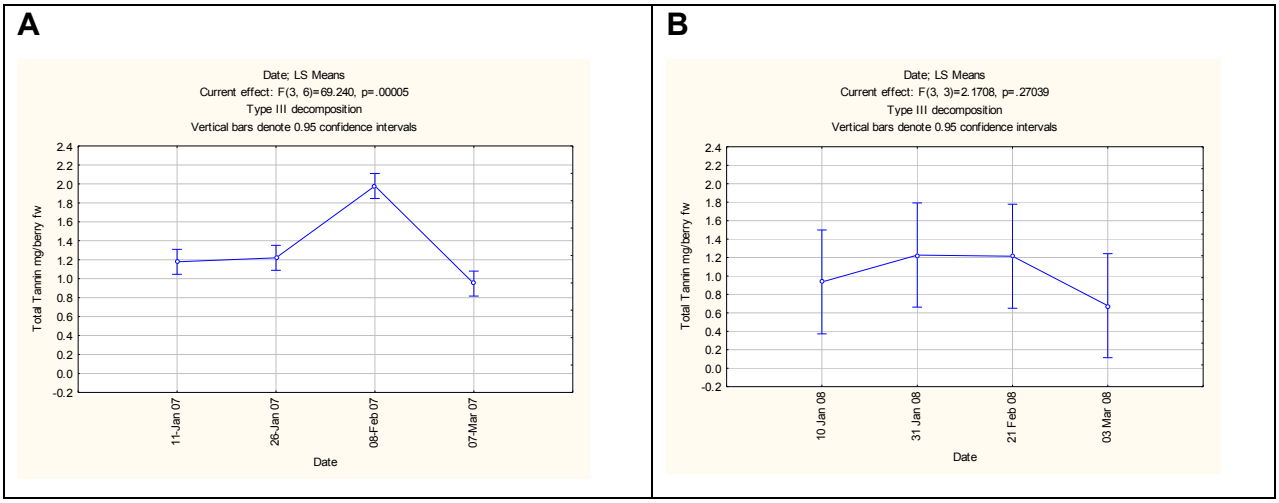


Figure 4.18 Total tannin content per berry fw at four ripening stages during the A. 2006/2007 (pre-veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) and B. 2007/2008 (pre-veraison 10 Jan 08; post- veraison 31 Jan 08; post-veraison 21 Feb 08; harvest 3 Mar 08) seasons respectively.

Anthocyanin levels (mg/berry fw) increased significantly from pre-veraison to harvest; although for the 2007/2008 season there was a significant decrease in anthocyanin from post-veraison to harvest (Figure 4.19). This indicates that in the 2007/2008 season the grape berries were becoming overripe with a breakdown of anthocyanin (Ribéreau-Gayon *et al*. 2000) No significant differences were observed at harvest between the two seasons (Table 4.3).

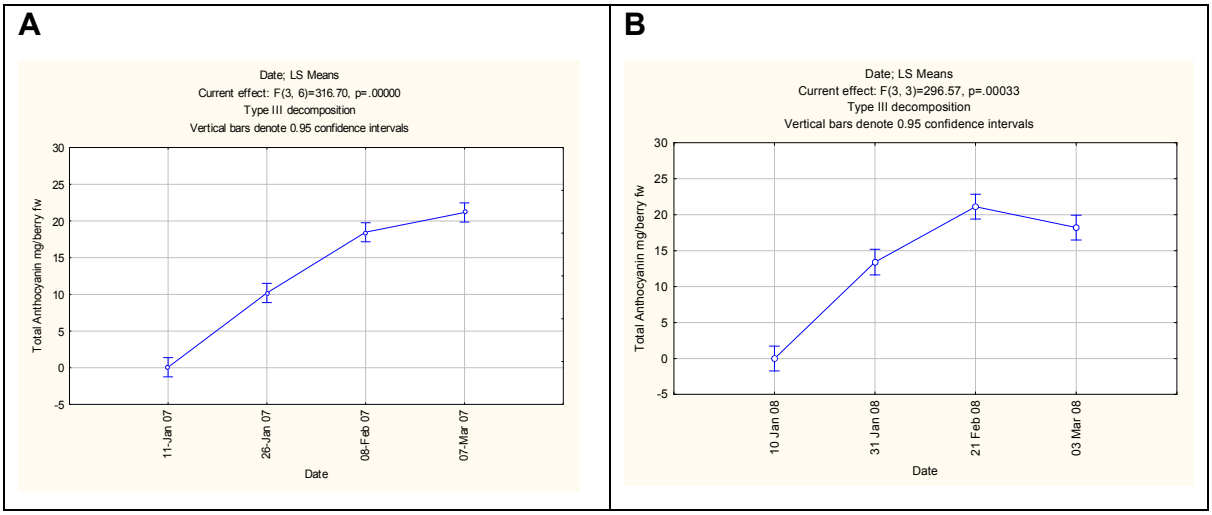


Figure 4.19 Total anthocyanin content per berry fw at four ripening stages during the A. 2006/2007 (pre-veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) and B. 2007/2008 (pre-veraison 10 Jan 08; post- veraison 31 Jan 08; post-veraison 21 Feb 08; harvest 3 Mar 08) seasons respectively.

It appears that the tannin and malic acid content of berries were more sensitive to the influence of the different climatic conditions between the two seasons studied. With more malic acid present in the berries of the 2007/2008 ripening season which was a wetter season with more frequent rain and higher temperatures closer to harvest. On the other hand higher concentrations of tannin were observed for the 2006/2007 ripening season which was a dryer season pre-veraison with large amounts of rain close to harvest.

Regarding the carotenoid content for the seasons studied, the carotenoid zeaxanthin was present in very small amounts in grape berries and degraded as ripening progressed. Pre-veraison zeaxanthin was present in berries at levels of 0.15 to 0.3 µg/berry fw and decreased to negligible amounts to 0.050 µg/berry at harvest (Appendix B, Figure 10A). Significant differences were observed at harvest between the two seasons studied with an average value of 0.01 µg/berry for the 2006/2007 season while the 2007/2008 had an average value of 0.03 µg/berry fw (Table 4.3).

5,8-Epoxy-β-carotene, an oxidation product of β-carotene, accumulated from pre-veraison to post-veraison where after it decreased as ripening progressed. 5,8-Epoxy-β-carotene behaved differently to most of the other carotenoids and chlorophylls which decreased from earlier in the season until harvest. This carotenoid appears to be very sensitive to climatic differences. The grape berries from the 2007/2008 season contained (1.55 µg/berry) five times more 5,8-epoxy-β-carotene compared to the berries of the 2006/2007 (0.28 µg/berry) season at harvest (Appendix B, Figure 10B). 5,8-Epoxy-β-carotene represents approximately 30% of the total concentration of carotenoids per berry fw in the 2007/2008 season at harvest. In the 2006/2007 season only 12% of the total carotenoid concentration was presented by 5,8-epoxy-β-carotene at harvest. This compound has been detected previously by Mendes-Pinto *et al.* (2004) as an unknown compound in grape extracts from cvs. Tinta Barroca, Touriga Francesa and Tinta Roriz but has not been quantified.

Lutein, a well know carotenoid present in grape berries showed an increase in the first part of the ripening (pre-veraison to post-veraison) season starting at levels of 1.6 to 2.6 µg/berry pre-veraison, peaking at 2 to 3 µg/berry and decreasing to 1.0 to 1.8 µg/berry at harvest (Appendix B, Figure 10C). The 2006/2007 season had significantly lower levels of lutein (1.55 µg/berry fw) compared to the 2007/2008 season average of 1.72 µg/berry at

harvest. Similar amounts of lutein in grape berries were found by De Pinho *et al.* (2001). Razungles *et al*. (1988, 1996) found a decrease in lutein content of berries from veraison to harvest.

β-carotene appears to be less sensitive to differences in climatic conditions since no significant differences in β-carotene per berry fw were observed at harvest between the two seasons studied (Table 4.3). No significant increases of β-carotene concentration per berry fresh weight were observed during the ripening seasons but significant decreases were evident from post-veraison to harvest as found by Razungles *et al*. (1988, 1996). At harvest an average β-carotene concentration of 0.62 µg/berry fw was found for the Merlot berries for both seasons studied. Similar concentrations of β-carotene were also found by De Pinho *et al.* (2001) in grape berries.

The *cis*-isomer of β-carotene was present in approximately ten times smaller quantities per berry fw at harvest than β-carotene. *Cis* β-carotene showed significantly different concentrations per berry fw at harvest between the two seasons studied. Grape berries from the 2006/2007 season contained 0.05 µg/berry fw *cis* β-carotene while the latter season's berries contained an average value of 0.08 µg/berry fw. *Cis*-isomers of β-carotene have been reported in grapes previously although it is still uncertain if they are an artefact of sample preparation and analysis (Mendes-Pinto 2004).

β-carotene and lutein were the most common carotenoids found in mature Merlot berries representing more than 80% of the total portion of carotenoids analysed per berry fresh weight in the 2006/2007 season. This is in agreement with what other researchers have found (Baumes *et al.* 2002; Marais *et al.* 1990, Oliveira *et al.* 2004; Razungles *et al.* 1988, 1998). However in the 2007/2008 season β-carotene and lutein represented only approximately 50% of the total carotenoids at harvest because of the high contribution of 5,8-epoxy-β-carotene.

The total carotenoid levels for the 2007/2008 season (4.49 µg/berry) at harvest was almost double the content found for the 2006/2007 season (2.61 µg/berry) (Table 4.3). This result is mainly due to the significant increase of 5,8-epoxy-β-carotene in the 2007/2008 season.

One would expect a decrease in total carotenoids in the 2007/2008 season because of the higher temperature during the harvest period which might have favoured degradation of carotenoids, but this was not the case. However Rodriguez-Amaya *et al.* (2008) stated that

warmer temperatures and greater exposure to sunlight increase cartenogenesis (synthesis of carotenoids), but may also promote carotenoid photo-degradation. It was found from studies in Brazil that papayas, cherries and mangoes of the same cultivars produced in hot regions contained distinctly higher carotenoid concentrations than those in temperate climates (Rodriguez-Amaya *et al.* 2008).

Although no significant difference in the amount of rainfall between the seasons was measured it is evident from figure 4.13 that small amounts of rain fell more frequently in the 2007/2008 ripening season compared to the 2006/2007 season. For the 2006/2007 ripening season more frequent decreases in solar radiation was evident which might be due to more cloudy weather conditions (Figure 4.5). Düring and Davtyan (2002) showed in their work that the xanthophyll pool size decreased for both cultivars Kerner and Portugieser during a rain period. It was suggested that the xanthophyll pool size adjusted according to the ambient conditions.

Moreover, Demmig-Adams *et al.* (1996) discussed in his review the time scale in which reactions in the xantophyll cycle takes place and it varied from a few minutes (de-epoxidation) to hours (epoxidation) in response to various environmental conditions. Thus the environmental conditions under which grape samples were collected might have had an effect on the carotenoid content of the berries which were analysed.

Chlorophyll a (sum of chlorophyll a and pheophytin a), was found to be the most abundant pigment present in Merlot grape berries throughout the ripening season. However significant decreases could be observed from post-veraison to harvest (Appendix B, Figure 11A). Pre-veraison, chlorophyll a was present in concentrations of 20 to 30 µg/berry and degraded towards harvest to 0.6 to 10 µg/berry (Appendix B, Figure 11A). Significant differences between the chlorophyll a content for the berries from the 2006/2007 (11.74 µg/berry) and 2007/2008 (9.02 µg/berry) seasons were observed. The chlorophyll a concentrations found in this study was 60 times more compared to chlorophyll a concentrations reported by Oliveira *et al.* (2003) in berries of cv. Touriga Nacional. These large differences can be explained by cultivar and terroir differences as well as the fact that in the current study chlorophyll a concentration was determined by sum of chlorophyll a and its derivatives. This was performed because it was shown that chlorophyll a is degraded by low pH of berries during extraction, as discussed in Chapter 3.

Chlorophyll b (sum of chlorophyll b, pheophytin b and pyropheophytin b) was present in berries in the beginning of the season at values of 5 to 8 µg/berry and degraded to 1 to 3

µg/berry by harvest (Appendix B, Figure 11B). An increase of chlorophyll b from pre-veraison to veraison (26 Jan 07) (2006/2007) and post-veraison (31 Jan 08) (2007/2008) were observed, where after it decreased to harvest. Significantly lower amounts of chlorophyll b at harvest was observed in the 2006/2007 (2.50 µg/berry) season compared to the 2007/2008 season (3.84 µg/berry).

The chlorophyll a content was four times that of chlorophyll b measured pre-veraison per berry. At harvest, chlorophyll b was present at 20 to 30% of its original concentration. Chlorophyll a, however was only reduced to 50% of the initial amount that was observed pre-veraison per berry fw by harvest. Giovanelli and Brenna (2007) studied chlorophyll during ripening of two red cultivars Barbera and Nebbiolo and found 14 to 20% of the initial concentration of chlorophyll at berry maturity. Giovanelli and Brenna (2006) found that chlorophyll a was up to ten times more concentrated at the beginning of berry development. While Gross (1991) stated that in higher plants chlorophyll a and chlorophyll b exist in a ratio of approximately three to one but this value can vary with growth and environmental conditions.

Mesoclimatic differences between seasons may thus be a potential reason for the significant differences observed in carotenoid and chlorophyll concentration per berry for the 2006/2007 and 2007/2008 ripening seasons.

4.3.5 PREDICTION AND EXPLORATION OF CAROTENOID AND CHLOROPHYLL CONCENTRATION IN GRAPES WITH REGARDS TO RIPENING MEASUREMENTS

In this section the possibility of using a within-vineyard model to predict optimal ripeness from carotenoid and chlorophyll measurements was explored for both seasons. Multivariate analysis (chemometrics) was a valuable tool in exploring the large data set with a lot of variables, and examining the potential of each to predict other variables in the dataset. PLS2 multivariate analysis allowed the interaction between the X and Y matrix and produces a visual interpretation of data showing possible correlations between compounds and the potential of the X-data matrix to predict data of the Y-matrix.

An X data matrix was constructed of grape samples of each experimental plot with sub-plot replicates at different ripening stages (veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) as objects and individual carotenoids and

chlorophylls per berry fresh weight as variables. The Y-data matrix consisted of grape samples of each plot with sub-plot replicates at the same ripening stages as for the X-matrix as objects and the ripening parameters (total glucose and fructose, malic acid, total tannin and total anthocyanin) as variables. Two outliers on PC 3 were removed from the model for the 2006/2007 season (sample replicate 2 and 3 of plot Mw 2 post-veraison). The PLS2 model using PC 1 and PC 2 explain 86% of the variance of the X matrix (chlorophyll and carotenoid data) data and 61% of the Y matrix (ripening parameters) data for the 2006/2007 model (Figure 4.15). This model shows good potential to predict ripening parameters over time with a correlation of 0.84 in Merlot berries per berry fresh weight for the 2006/2007 season.

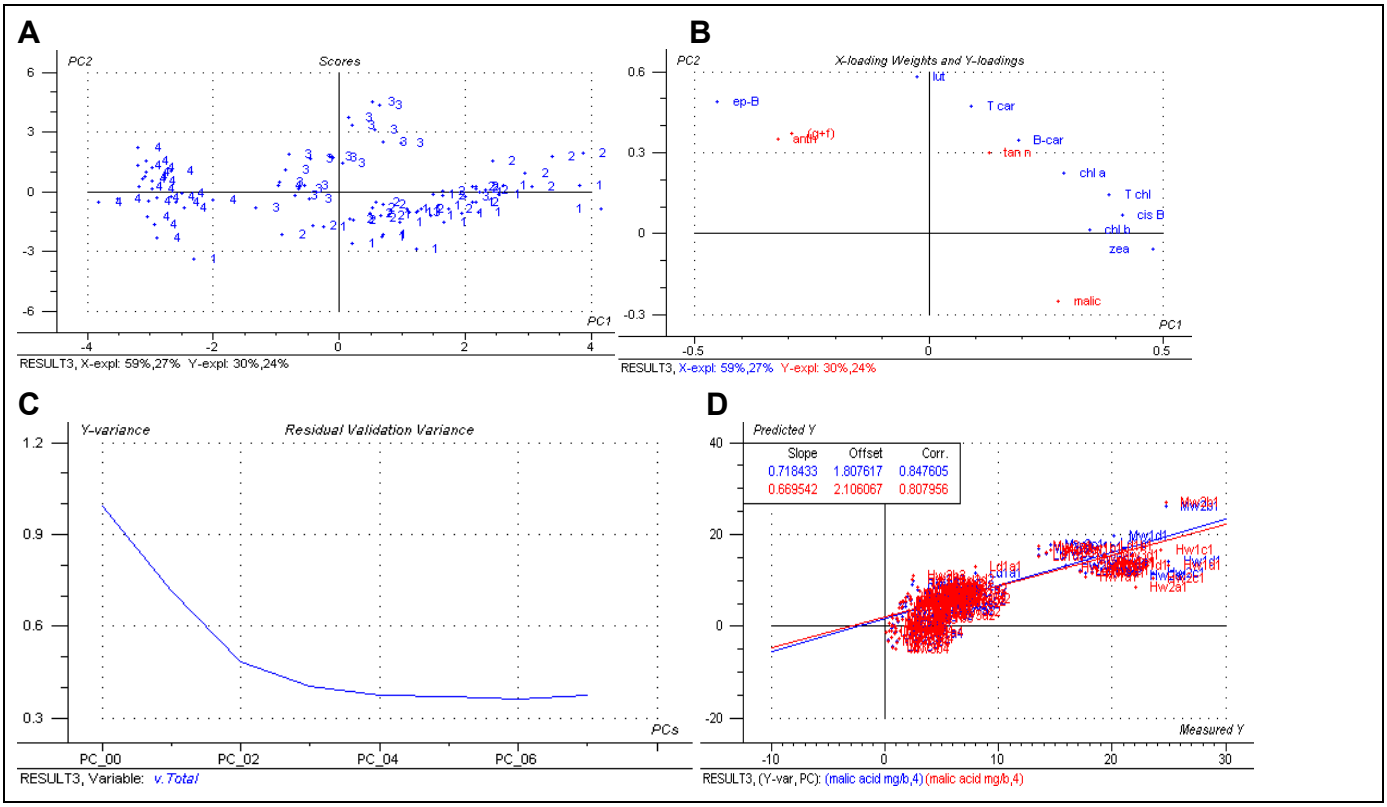


Figure 4.15 Preliminary model for the prediction of ripening parameters per berry fresh weight from chlorophyll and carotenoid content per berry for the 2006/2007 season. A. *Scores:* 1 (pre-veraison, 11 Jan 07); 2 (veraison, 26 Jan 07); 3 (post-veraison, 8 Feb 07); 4 (harvest, 7 Mar 07). B. *X and Y loadings weights:* 5,8-epoxy-β-carotene (ep-B); lutein (lut); β-carotene (B-car); chlorophyll a (chl a); *cis*- β-carotene (cis-B); zeaxanthin (zea); chlorophyll b (chl b); anthocyanin (anth); Total tannin (tann); Total glucose and fructose (g+f); malic acid (malic). C. *Residual Validation Variance.* D. *Predicted Y.*

A similar ripening model to the 2006/2007 season model was constructed for the 2007/2008 season. PC 1 and PC 2 explain 90% of the X matrix variance (carotenoid and chlorophyll data) and 48% of the Y matrix (ripening parameters) variance (Figure 4.16). The model shows potential similar to the 2006/2007 season to predict ripening parameters over time with a correlation of 0.84 in Merlot berries per berry fresh weight.

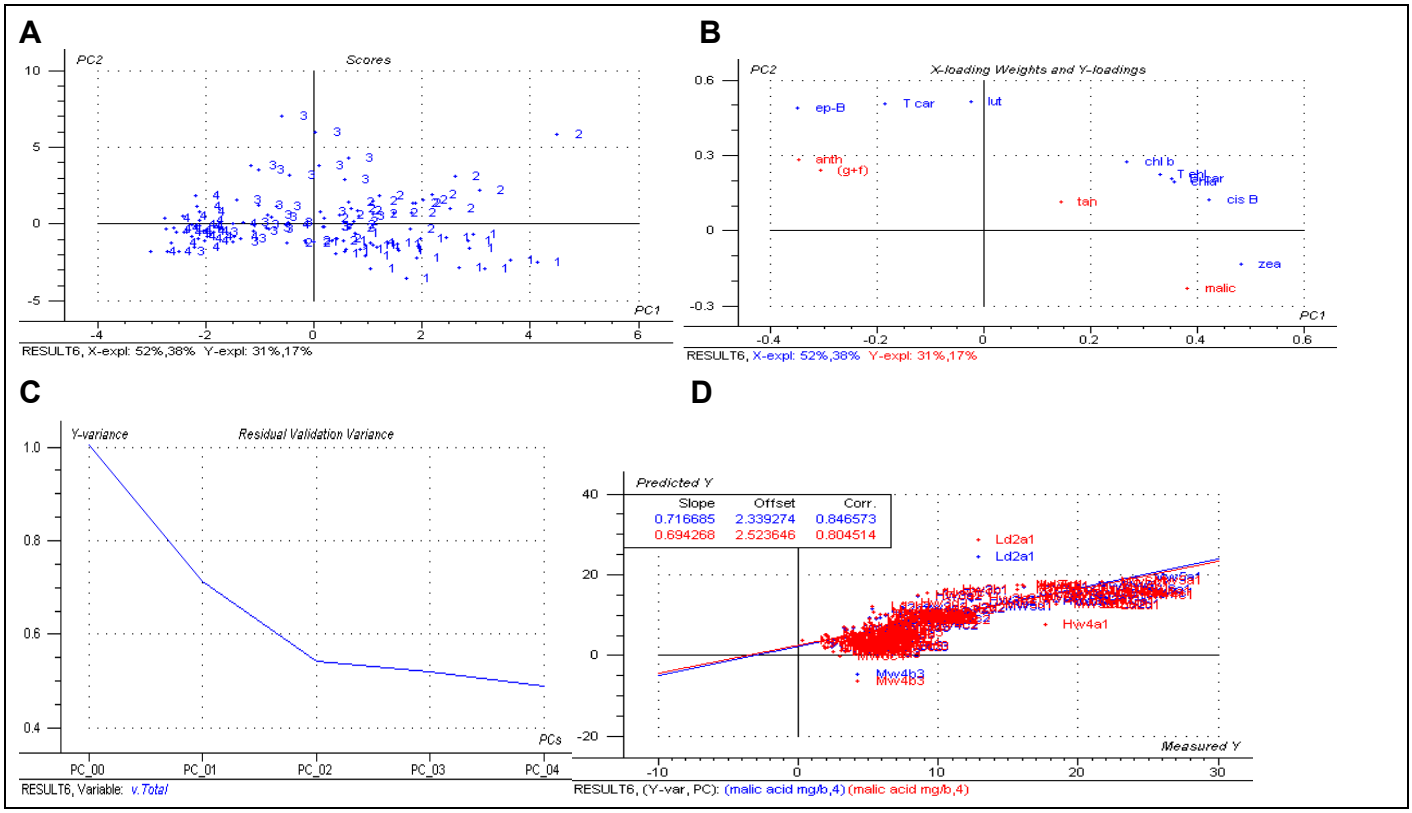


Figure 4.16 Preliminary model for the prediction of ripening parameters per berry fresh weight from chlorophyll and carotenoid content per berry for the 2008 season. A. *Scores:* 1 (pre-veraison, 10 Jan 08); 2 (post-veraison, 31 Jan 08); 3 (post-veraison, 21 Feb 08); 4 (harvest, 3 Mar 08). B. *X and Y loading weights:* 5,8-epoxy-β-carotene (ep-B); lutein (lut); β-carotene (B-car); chlorophyll a (chl a); *cis*- β-carotene (cis-B); zeaxanthin (zea); chlorophyll b (chl b); Total carotenoids (T car); Total; chlorophyll (T chl); anthocyanin (anth); Total tannin (tann); Total glucose and fructose (g+f); malic acid (malic). C. *Residual Validation Variance.* D. *Predicted Y.*

These results thus indicate the great potential of carotenoids and chlorophylls to be used in the future to predict optimal ripeness. However a lot of research is still necessary to elucidate on the application of such models.

Moreover, correlations between individual carotenoids, chlorophylls and ripening parameters determined as content per berry fw were evaluated by normal regression

statistics which showed only a few significant (r≥0.5) correlations (Appendix B, Table 10 and 11). Zeaxanthin showed for both seasons a positive correlation with malic acid with r values of 0.5 and negative correlations with total glucose and fructose and anthocyanin with r values greater than -0.6 for the 2006/2007 season and -0.5 for the 2007/2008 season respectively. However, from the preliminary prediction models (Figure 4.15 and 4.16) it appears that the profile of carotenoids and chlorophylls in berries together describe (predict) the concentrations of ripening parameters through ripening without strong correlations between individual compounds. For these results to take application in the industry a suitable device to accurately and non-destructively measure the carotenoid and chlorophyll content of berries will be needed.

Research by Kolb *et al*. (2006) indicated that chlorophyll fluorescence measurements are well-suited to determine non-invasively sugar accumulation in white grape berries cv. Bacchus and Silvaner. Furthermore, Agati *et al.* (2008) showed that a chlorophyll fluorescence imaging method based on pigment screening of excitation is able to determine the distribution of anthocyanin in whole grape bunches. These studies show the potential of using chlorophyll fluorescence measurements to predict ripening. Furthermore such measurements coupled with chemometric analysis can generate valuable visual interpretations of the relation of pigment data with ripening variables. For example Le Moinge *et al.* (2008) showed in his study, that front face fluorescence spectroscopy and visible spectroscopy coupled with chemometrics has the potential to characterise ripening of Cabernet Franc grapes. Pereira *et al.* (2006) showed by using ^{1}H NMR spectroscopy together with chemometric data analyses that vintage effects on grape metabolic profiles prevail over soil effects.

Another technique to measure pigments is near infrared spectroscopy (NIR). Research has shown that NIR-FT-Raman (near infrared fourier transform) spectroscopy can give a sensitive detection of the individual carotenoids by Raman Resonance in the visible region (Withnall *et al.* 2003; Veronelli *et al.* 1995). Raman is a spectroscopic technique used in condensed matter physics and chemistry to study the vibration, rotation, and other low-frequency modes in a system (Gardiner 1989). FT-Raman spectroscopy can also gives a strong enhancement of carotenoids due to the known pre-resonance effects. In addition the disturbing fluorescence effect of biological material usually observed when laser excitation is performed in the visible wavelength range can be avoided (Ozaki *et al*. 1992). Strong bands of carotenoids are observed in the Raman spectrum within the 1500-1550 and 1150-

1170 cm^{-1} range due to in-phase C=C and C-C stretching vibrations of the polyene chain (Withnall *et al.* 2003; Veronelli *et al*. 1995). It has been found that FT-Raman spectroscopy can be successfully applied for the identification of carotenoids directly in the plant tissue without any preliminary sample preparation. Furthermore, FT-Raman mapping is able to show the location of carotenoids in the surface layer of the plant tissue and perform semi-quantitative measurements of these carotenoids (Schultz *et al.* 2005).

4.4 CONCLUSION

This study confirmed that in general carotenoids and chlorophylls decrease on a per berry (µg/berry) and concentration (µg/g) basis from veraison to harvest. However this study also found that vigour differences might have an effect on the rate of synthesis/degradation of carotenoids, chlorophylls and some other ripening parameters (malic acid, total glucose and fructose, total tannin and total anthocyanin from pre-veraison (pea size) to harvest in berries on a per berry basis not necessarily causing significant differences in content at harvest. The effect of soil water content, and other field variables influenced by this measure on carotenoids, chlorophylls and ripeness parameters were not significant in this study because of high soil water capacity of lower soil layers which prevented significant differences in water deficits. 5,8-Epoxy-β-carotene was quantified for the first time in grapes and represents a significant amount of total carotenoids at harvest. All the carotenoids and chlorophylls except β-carotene seemed to be sensitive to annual climate condition differences. Lutein and β-carotene were found to be the most abundant carotenoids present in Merlot grape berries together with chlorophyll a for both seasons studied. The values of these carotenoids also correlated well with previous research. However, chlorophyll a was found in much larger quantities in Merlot berries compared to previous research. This is possibly because in this study the chlorophyll degradation products where included in the calculation of chlorophyll a.

Multivariate analysis showed promising preliminary prediction models (with correlation values of above 0.8 for both seasons analysed) for the prediction of the concentration of ripeness parameters (glucose, fructose, malic acid, total tannins and anthocyanins) with carotenoids and chlorophyll content. This result highlights the opportunity for the development of a rapid non-destructive method to measure carotenoids and chlorophylls in

berries which in turn can predict optimal ripeness. Furthermore since carotenoids are the precursors to C_{13}-norisoprenoid aroma compounds in wine a preview of the potential contribution of these aromas to wine might be evaluated. Further research is necessary to investigate the possibility of building and validating such models.

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Chapter 5

General Discussion and Conclusions

GENERAL DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSIONS AND CONCLUSIONS

In this study, some important contributions have been made regarding the conditions for the extraction of carotenoids and chlorophylls from grape tissues, as well as optimisation of the HPLC method for the simultaneous detection and quantitation of these pigments in extracts. For the extraction of chlorophylls and carotenoids, the importance of pH was highlighted in this study, and significant degradation of both pigment types was observed under low pH. This was found to be more significant for green grape berry tissue when compared to red berry tissue, and was due to higher acid levels in the former. A thorough review of the literature to date showed that few studies have mentioned the use of buffer solutions during carotenoid and chlorophyll extraction. The results of this study have shown that this can cause incorrect interpretation analytical data if the pH of the final extract was not considered. The experimental results showed that the use of TRIS buffer limited the extractability of certain carotenoids which were of interest to the research question at hand. As such, the extraction conditions were not buffered for the experiments performed, but it is proposed that in future research the extraction method for grape berry tissue at different stages of ripeness should be optimised further to effectively neutralise tissue acidity, without compromising the extraction of carotenoids. A further question raised by the research results was as to whether *cis*-isomers and chlorophyll degradation products are naturally present in grape berries, or are formed during sampling and processing. This was not addressed in the current study.

A significant finding was that 5,8-epoxy-β-carotene was identified in grape berry tissue for the first time, thus broadening the range of detectable compounds in grape berries. However, previous research has shown unidentified compounds with similar spectra and elution times in grape berries (Mendes-Pinto et al. 2005, Mendes-Pinto et al. 2004). 5,8-epoxy-β-carotene is also present in a Brazilian tropical fruit camu-camu (*Myrciaria dubia*) (Zanatta and Mercadante 2007). Additionally, 5,8-epoxy-β-carotene and its 5,6-epoxide isomers can be found in a variety of plants, although it is not certain if 5,8-poxy-β-carotene is an artefact formed from 5,6-epoxide via epoxide-furanoid rearrangement during the extraction process (Deli and Ozs 2004). Little is known and reported on the evolution of 5,8-epoxy-β-carotene in fruits during ripening. However 5,8-epoxy-β-carotene differed from the other grape carotenoids in this study since this

compound increased with berry ripening while the other grape carotenoids decreased with maturity. 5,6–epoxides can rearrange to form 5,8-epoxy-β-carotene *in vitro* on treatment with diluted acids (Deli and Ozs 2004). This might be an indication as to why this compound was present in green berry tissue which contains a higher relative acidity than ripe grape tissue. However, 5,8-epoxy-β-carotene showed an increase with ripening although the acid concentration decreased. Its presence may therefore not be due to the acidic extraction conditions alone, but this was not conclusively shown within the scope of this study. More research is necessary in order to understand the evolution and *in vitro* rearrangement of this compound during grape maturation.

Furthermore, this research has confirmed previous observations that, in general, carotenoids and chlorophylls decrease on a per berry (µg/berry) and concentration (µg/g) basis from veraison to harvest. The research results were inconclusive in addressing the research question, such that vigour differences had little effect on the rate of synthesis and/or degradation of carotenoids, chlorophyll and some other ripening parameters, namely malic acid, hexose sugars, tannin and anthocyanin from pre-veraison (pea size) to full ripeness. Additionally, no significant effect of soil water content on carotenoids, chlorophylls and ripeness parameters was found in this study, most likely due to the fact that high soil water capacity was found in lower soil layers which minimized differences in grapevine water status although the irrigation water applied was varied significantly in the field experiment. According to the literature to date, under warmer conditions, with higher sunlight intensity, less dense grapevine canopies will obtain unripe, sun-exposed berries which are better adapted to higher light intensities than shade-adapted berries due to their higher capacity for photosynthetic energy consumption and thermal energy dissipation (Düring and Davtyan 2002). Furthermore, a higher xanthophyll pool can be expected under clear, warm-weather conditions before or at veraison while in shade-adapted/exposed berries the xanthophyll pool size can decrease to lower initial pool levels before or at veraison (Düring and Davtyan 2002). Under lowered soil water conditions with low soil water-retention (sandy soil) carotenoid content can increase up to 60% while high water-retention capacity soil, shows no effect (Oliveira *et al.* 2003). The response of carotenoids to water stress occurs in fruit from an early stage of development, and the effect on carotenoid content can be retained as the fruit matures (Oliveira *et al.* 2003). To summarise, it appears that a warmer climate with higher amount of sunlight incidence and low water-capacity soils might lead to berries with a greater pre-veraison xanthophyll pool size. However more research is necessary to evaluate the threshold values when these occurrences

take effect together with the impact on other significant compounds with respect to grape aroma potential, namely the C_{13}-norisoprenoids.

Due to the sensitivity of carotenoids and chlorophylls to degradation, uncertainties exist as to whether analysis of these compounds with current HPLC methods, which require extensive sample processing and extraction, are entirely representative of the *in vivo* content of these compounds in grape berries. In addition, this labour-intensive and expensive process limits the extent to which photosynthetic pigments can be monitored in response to viticultural research questions. A possible solution would be the development of a device to non-invasively and accurately quantify carotenoids and chlorophyll in berries. Furthermore, viticulturists might benefit from such a device to monitor ripeness since it has been shown that carotenoids and chlorophylls are potential ripeness indicators, and may be more sensitive indicators of the progress of ripening than traditional measures such as titratable acidity, pH, total soluble solids or anthocyanin (Lund *et al.* 2008). Preliminary work on the relation of some individual carotenoids and chlorophylls with other ripeness parameters has been shown in this study. Zeaxanthin determined as content per berry fresh weight showed for both seasons a positive correlation with malic acid with r values of 0.5 and negative correlations with total glucose and fructose and anthocyanin with r values greater than -0.6 for the 2006/2007 season and -0.5 for the 2007/2008 season respectively. However, from the preliminary prediction models (Figure 4.15 and 4.16) it appears that the profile of carotenoids and chlorophylls in berries together describe (predict) the concentrations of ripening parameters through ripening without strong correlations between individual compounds. The potential of carotenoids and chlorophylls to predict berry ripeness were investigated with multivariate analyses and showed correlations of more than 0.8 for both seasons studied. However, extensive research is still necessary to evaluate such an application. A suitable device to accurately and non-destructively measure the carotenoid and chlorophyll content of berries will be needed for these results to take application in the industry. The relationship of carotenoid and chlorophyll profiles to ripeness also needs to be established further since little research has been done in this field also evaluating different cultivars and climates.

Carotenoids degrade during berry ripening, and the current theory suggests that they are enzymatically cleaved to give rise to C_{13}-norisoprenoid precursors in grape tissue (Baumes *et al.* 2002). Under low pH conditions during vinification, C_{13}-norisoprenoids are generated from their carotenoid precursors and, contribute floral (Kanasawud and Crouzet 1990; Kovats 1987; Ohloff 1978) and honey-like aromas to

the wine. Since these volatile aroma compounds (C_{13}-norisoprenoids) can not be measured directly in grapes during maturation, and previous research has correlated the rate of carotenoid degradation to the evolution of C_{13}-norisoprenoid precursors, carotenoids might give a valuable indication of their rate of formation. This connection between the degradation of carotenoids and the formation of flavour compounds has been partially studied and needs to be established in the future. However, a significant limitation in this research direction is the analytical methods required, which are costly and labour intensive.

A non-invasive device to measure carotenoids in fruit was already patented in 2004 by Gellerman *et al.* (2004). This portable non-invasive device uses Raman scattering spectroscopy of carotenoids as indication of oxidative deterioration which gives an indication of the general health status of higher plants. Unfortunately no no-invasive devices are currently availably to accurately quantify carotenoids and chlorophyll content of berries. Thus, there is scope for further research to improve the analytical measurement of carotenoids and chlorophylls to obtain maximum information from these pigments in relation to viticultural variables, and to serve as a basis for the calibration and validation of non-invasive methods for pigment detection and quantitation.

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Appendix A

Table 1 Determination of carotenoids and chlorophylls and their derivatives in grape berries for the 2006/2007 ripening season.

Maturation stage	Date	Plot code	Weight per berry (g)	cis Neo	Pyropheo b	Chl b	Lut	Mutatox	Zea	ep β-car	Chl a	Pheophor b	Pheo b	Pheo a	β-car	cis β-car	Total car	Total chl	Juice pH
				**Average µg/g fresh weight															
Pre-veraison	11-Jan-07	Hw 1	0.862	0.136	1.650	0.304	2.025	0.000	0.175	0.041	0.000	0.326	7.330	27.184	1.126	0.145	3.705	36.793	*nm
	11-Jan-07	Hw 2	0.755	0.260	3.240	0.779	3.020	0.026	0.359	0.065	0.000	2.965	7.567	33.161	1.683	0.211	5.697	55.384	nm
	11-Jan-07	Mw 1	0.772	0.205	3.977	0.829	2.377	0.000	0.244	0.065	0.000	0.963	6.737	35.185	1.072	0.175	4.701	45.411	nm
	11-Jan-07	Mw 2	0.797	0.186	2.288	0.786	2.164	0.000	0.172	0.045	0.000	0.766	6.418	28.425	1.240	0.121	3.985	38.682	nm
	11-Jan-07	Mw 3	0.819	0.110	4.061	0.275	2.238	0.000	0.242	0.099	0.000	0.496	6.038	28.593	1.249	0.163	4.124	39.463	nm
	11-Jan-07	Ld 1	0.657	0.193	0.991	1.009	1.928	0.000	0.222	0.026	0.000	0.454	6.397	26.223	1.141	0.138	3.806	35.074	nm
Veraison	26-Jan-07	Hw 1	1.345	0.185	0.317	0.973	1.565	0.000	0.146	0.022	0.000	1.309	3.430	16.875	0.773	0.087	2.802	22.904	3.17
	26-Jan-07	Mw 1	1.117	0.080	0.399	2.360	1.765	0.000	0.133	0.050	0.000	0.173	2.286	15.078	0.887	0.098	3.037	26.166	3.16
	26-Jan-07	Mw 3	1.374	0.168	0.252	0.631	1.844	0.000	0.151	0.127	0.000	1.116	4.717	19.450	0.918	0.111	3.375	27.327	3.15
	26-Jan-07	Mw 2	1.291	0.043	0.163	0.401	1.599	0.000	0.095	0.028	0.000	0.960	4.519	17.855	0.827	0.099	2.728	23.898	3.13
	26-Jan-07	Hw 2	1.094	0.196	0.354	0.299	1.386	0.024	0.096	0.000	0.000	3.267	3.767	19.638	0.697	0.081	2.456	20.296	3.15
	26-Jan-07	Ld 1	1.125	0.021	0.000	0.163	1.577	0.019	0.147	0.031	0.000	1.243	4.565	17.262	0.761	0.094	2.674	23.232	3.14
Post-veraison	8-Feb-07	Hw 1	1.459	0.066	5.535	0.057	1.714	0.000	0.093	0.339	0.000	0.000	1.756	16.948	0.830	0.078	3.138	24.296	3.41
	8-Feb-07	Mw 1	1.382	0.033	0.000	0.061	1.489	0.000	0.067	0.299	0.000	4.023	4.367	10.848	0.729	0.074	2.712	22.212	3.42
	8-Feb-07	Mw 3	1.505	0.058	0.000	0.053	1.391	0.000	0.067	0.213	0.000	5.496	1.261	15.402	0.662	0.062	2.454	19.687	3.37
	8-Feb-07	Mw 2	1.396	0.028	5.509	0.031	1.560	0.000	0.072	0.316	0.000	0.000	1.673	16.668	0.699	0.087	2.763	23.881	3.43
	8-Feb-07	Hw 2	1.423	0.026	3.335	0.097	1.244	0.000	0.084	0.039	0.000	0.263	1.976	14.015	0.551	0.095	3.336	19.300	3.39
	8-Feb-07	Ld 1	1.294	0.122	1.023	0.869	1.313	0.000	0.076	0.144	0.000	1.546	1.348	12.909	0.609	0.061	2.326	17.695	3.39
Harvest	7-Mar-07	Hw 1	1.503	0.076	0.945	0.248	1.080	0.000	0.031	0.140	0.000	0.300	1.361	8.014	0.428	0.042	1.798	10.867	3.6
	7-Mar-07	Mw 1	1.410	0.066	1.444	0.110	1.137	0.000	0.000	0.211	0.000	0.288	1.043	7.444	0.474	0.034	1.924	11.715	3.53
	7-Mar-07	Mw 3	1.561	0.032	1.528	0.402	1.023	0.003	0.000	0.187	0.000	0.376	0.840	8.569	0.405	0.041	1.687	10.393	3.59
	7-Mar-07	Mw 2	1.569	0.038	2.230	0.038	0.991	0.000	0.000	0.246	0.000	0.000	0.981	7.981	0.401	0.031	1.706	11.230	3.6
	7-Mar-07	Hw 2	1.502	0.069	1.187	0.039	0.955	0.000	0.000	0.183	0.000	0.159	1.330	7.677	0.387	0.030	1.625	10.329	3.64
	7-Mar-07	Ld 1	1.403	0.084	0.185	0.170	1.075	0.024	0.000	0.173	0.000	1.342	1.125	7.626	0.409	0.040	1.806	10.449	3.54

**Average (Ave) calculated from 4 biological replicates which was analysed in triplicate (standard error ≤ 25%) standard error for derivatives not calculated.
* Not measured (nm)
Carotenoids and chlorophylls: cis-Neoxanthin (cis Neo); Pyropheophytin b (Pyropheo b); Chlorophyll b (Chl b); Lutein (Lut); Mutatoxanthin (Mutatox); Zeaxanthin (Zea); 5,8-epoxy-β-carotene (ep- β-car); Chlorophyll a (Chl a); Pheophorbide b (Pheophor b); Pheophytin b (Pheo b); Pheophytin a (Pheo a); β-carotene (β-car); *cis*- β-carotene (*cis*-β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds (see chapter 3)
Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1; Ld2) (See chapter 4 for more detail).

Table 2 Determination of carotenoids and chlorophylls and their derivatives in grape berries for the 2007/2008 ripening season.

Maturation stage	Date	Plot code	Weight per berry (g)	cis Neo	Pyropheo b	Chl b	Lut	Mutatox	Zea	ep β-car	Chl a	Pheophor b	Pheo b	Pheo a	β-car	*cis* β-car	Tot car	Tot chl	Juice pH
			Average µg/g fresh weight																
Pre-veraison	10-Jan-08	Hw 3	0.730	0.463	1.362	4.992	2.874	0.042	0.320	0.333	0.678	0.926	4.616	25.523	1.576	0.218	6.021	38.097	*nm
	10-Jan-08	Hw 4	0.782	0.357	1.108	2.966	2.505	0.072	0.430	0.212	0.199	0.788	5.560	17.919	1.414	0.228	4.037	26.531	nm
	10-Jan-08	Hw 5	0.681	0.437	1.538	4.859	2.990	0.052	0.127	0.215	0.542	0.827	5.306	26.929	1.679	0.220	5.890	40.000	nm
	10-Jan-08	Mw 4	0.746	0.355	0.968	3.497	2.613	0.050	0.170	0.209	0.239	0.774	6.420	25.497	1.479	0.213	4.103	37.396	nm
	10-Jan-08	Mw 5	0.724	0.483	1.707	5.141	3.171	0.123	0.544	0.309	0.596	0.983	5.448	29.440	1.763	0.258	6.801	43.315	nm
	10-Jan-08	Mw 6	0.751	0.576	1.992	6.169	3.438	0.061	0.204	0.281	1.187	1.244	5.577	31.903	1.933	0.263	6.936	48.073	nm
	10-Jan-08	Ld 2	0.773	0.299	0.599	2.670	1.975	0.099	0.360	0.195	0.331	0.642	5.596	21.573	1.304	0.177	4.502	31.412	nm
Post-veraison	31-Jan-08	Hw 3	1.315	0.140	0.827	0.845	2.638	0.270	0.110	2.697	0.000	6.433	3.055	21.399	1.209	0.172	7.600	32.558	3.37
	31-Jan-08	Hw 4	1.227	0.155	0.765	0.989	1.865	0.092	0.089	1.000	0.000	2.733	2.479	14.359	0.870	0.112	4.601	21.326	3.29
	31-Jan-08	Hw 5	1.203	0.134	1.570	1.901	2.248	0.106	0.121	1.220	0.000	2.258	2.120	16.847	1.053	0.143	5.327	24.696	3.19
	31-Jan-08	Mw 4	1.272	0.091	0.398	0.199	2.038	0.166	0.085	1.765	0.000	4.378	2.479	15.359	0.878	0.126	5.439	22.812	3.23
	31-Jan-08	Mw 5	1.277	0.127	0.610	0.399	2.275	0.177	0.090	2.006	0.000	5.209	2.149	16.747	0.995	0.129	6.106	25.114	3.49
	31-Jan-08	Mw 6	1.170	0.109	0.902	1.839	2.348	0.215	0.120	1.828	0.424	4.607	1.265	16.939	0.990	0.147	6.122	25.975	3.27
	31-Jan-08	Ld 2	1.268	0.102	0.831	0.285	2.527	0.237	0.097	2.359	0.000	5.823	2.867	19.689	1.150	0.151	6.967	29.494	3.21
Post-veraison	21-Feb-08	Hw 3	1.474	0.079	0.637	0.749	1.613	0.056	0.072	0.961	0.000	1.199	2.221	10.168	0.686	0.092	3.834	14.973	26.6
	21-Feb-08	Hw 4	1.335	0.024	0.767	0.116	1.664	0.118	0.068	1.478	0.000	3.187	1.786	10.325	0.660	0.084	4.396	16.181	3.53
	21-Feb-08	Hw 5	1.265	0.052	0.448	0.309	1.510	0.053	0.056	0.815	0.000	2.031	1.706	8.952	0.583	0.073	3.865	13.446	3.51
	21-Feb-08	Mw 4	1.301	0.010	0.455	0.133	2.722	0.648	0.061	5.831	0.000	6.746	1.102	15.927	0.992	0.119	11.026	24.362	3.54
	21-Feb-08	Mw 5	1.303	0.053	0.444	0.147	1.925	0.245	0.057	1.257	0.000	3.778	1.432	11.507	0.745	0.094	4.762	17.309	3.54
	21-Feb-08	Mw 6	1.242	0.008	0.076	0.065	1.575	0.145	0.075	2.298	0.000	2.691	1.434	8.862	0.588	0.085	5.142	13.128	3.48
	21-Feb-08	Ld 2	1.339	0.075	0.725	0.159	2.468	0.441	0.071	4.430	0.000	6.212	1.380	15.374	0.986	0.121	9.088	23.849	3.45
Harvest	3-Mar-08	Hw 3	1.389	0.145	0.770	1.533	1.279	0.035	0.023	0.620	0.000	0.475	0.792	7.529	0.531	0.066	2.853	11.100	3.56
	3-Mar-08	Hw 4	1.367	0.016	0.083	0.066	1.225	0.087	0.034	0.907	0.000	1.813	1.318	6.712	0.424	0.049	2.956	9.992	3.56
	3-Mar-08	Hw 5	1.236	0.010	0.116	0.085	1.597	0.134	0.000	1.549	0.000	2.440	1.197	7.975	0.557	0.068	4.230	11.813	3.56
	3-Mar-08	Mw 4	1.302	0.070	0.494	0.455	1.342	0.099	0.000	1.419	0.000	1.091	0.985	6.624	0.497	0.063	3.751	9.649	3.63
	3-Mar-08	Mw 5	1.307	0.073	0.435	0.504	1.274	0.078	0.059	1.264	0.000	0.906	1.062	6.549	0.456	0.061	3.480	9.455	3.57
	3-Mar-08	Mw 6	1.316	0.009	0.072	0.076	1.189	0.145	0.024	1.488	0.000	1.605	0.945	5.663	0.401	0.054	3.575	8.361	3.52
	3-Mar-08	Ld 2	1.422	0.008	0.069	0.064	1.218	0.063	0.025	1.159	0.000	1.619	1.489	6.880	0.443	0.053	3.168	10.122	3.51

**Average (Ave) calculated from 4 biological replicates which was analysed in triplicate (standard error ≤ 25%) standard error for derivatives not calculated.
* Not measured (nm)
Carotenoids and chlorophylls: cis-Neoxanthin (cis Neo); Pyropheophytin b (Pyropheo b); Chlorophyll b (Chl b); Lutein (Lut); Mutatoxanthin (Mutatox); Zeaxanthin (Zea); 5,8-epoxy-β-carotene (ep- β-car); Chlorophyll a (Chl a); Pheophorbide b (Pheophor b); Pheophytin b (Pheo b); Pheophytin a (Pheo a); β-carotene (β-car); *cis*- β-carotene (*cis*-β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds (see chapter 3)
Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1; Ld2) (See chapter 4 for more detail).

Appendix B

Table 1 Irrigation and rainfall during 2006/2007 season.

Ripening stage	Date	Litres of water irrigated per plot per dripper during the 2007 season						Rain mm
	Plot	A2 (Mw 2)	A4 (Hw 2)	A12 (Ld 1)	1 (Hw 1)	3 (Mw 1)	8 (Mw 3)	
Pre-veraison	5-Jan-07	15.6	15.6	15.6				
	11-Jan-07			44.2			44.2	
Veraison	18-Jan-07				1.7	1.7	1.7	2
	22-Jan-07						5.2	
	23-Jan-07	15.6	15.6	15.6				
	24-Jan-07				7.8	7.8		
Post-veraison	26-Jan-07	31.2	31.2	31.2				
	7-Feb-07	15.6	15.6	15.6				
Harvest	11-Feb-07							26
	15-Feb-07							2.5
	16-Feb-07	15.6	15.6	15.6				
	22-Feb-07	10.4	10.4	10.4				
	28-Feb-07	23.4	23.4	23.4				
	4-Mar-07							40
	Total liters	127.4	127.4	171.6	9.5	9.5	51.1	70.5

Plots 2006/2007 season: Irrigated plots (A2; A4; A12), minimal irrigated plots (1; 3; 8) Reclassification of 2006/2007 plots according to vigour (pruning mass per vine) and soil water measurements (total soil water measured at three different depths divided by three): High vigour, wet plots (Hw 1; Hw 2), medium vigour, wet plots (Mw 1; Mw 2; Mw 3), low vigour dry plot (Ld 1).

Table 2 Irrigation and rainfall during 2007/2008 season.

Ripening stage	Date	Litres of water irrigated per plot per dripper during the 2008 season							Rain mm
	Plot	A3 (Mw 4)	A9 (Hw 3)	A12 (Ld 2)	2 (Mw 6)	3 Hw 5)	5 (Hw 4)	8 (Mw 5)	
Veraison	18-Jan-08	15.6	15.6	15.6					
	19-Jan-08				15.6	15.6	15.6	15.6	
	22-Jan-08	18.2	18.2	18.2					
	23-Jan-08				18.2	18.2	18.2	18.2	
Post-veraison	29-Jan-08				7.8	7.8	7.8	7.8	
	30-Jan-08	15.6	15.6	15.6	7.8	7.8	7.8	7.8	
	6-Feb-08	26	26	26	2.6	2.6	2.6	2.6	
Harvest	9-Feb-08								28
	13-Feb-08	20.8	20.8	20.8	5.2	5.2	5.2	5.2	
	16-Feb-08								5
	20-Feb-08	15.6	15.6	15.6	2.6	2.6	2.6	2.6	
	27-Feb-08								18
	Total liters	111.8	111.8	111.8	59.8	59.8	59.8	59.8	51

Plots 2007/2008 season: Irrigated plots (A3; A9; A12), minimal irrigated (2; 3; 5; 8)
Reclassification of 2007/2008 plots according to vigour (pruning mass per vine) and soil water measurements (total soil water measured at three different depths divided by three): High vigour, wet plots (Hw 3; Hw 4; Hw 5), medium vigour, wet plots (Mw 4; Mw 5; Mw 6), low vigour dry plot (Ld 2).

Table 3a Mean grapevine response data per plot (24 vines) for the 2006/2007 and 2007/2008 seasons.

Season	Plot	Plot code	Pruning mass per vine kg	Mass per shoot g	Inter-node length (cm)	Shoot diameter (mm)	[v]Number of lateral shoots per main shoot	[v]Lateral shoot length per main shoot (cm)	Main shoot length (cm)	Number of shoots per vine	Canopy management practice
2006/2007	1	Hw 1	1.01ab	64.48a	8.14a	7.84b	0.21a	5.58a	87.79a	16.47ab	topped
2006/2007	A2	Hw 2	1.03b	63.78a	7.57ab	5.05c	0.17a	3.25a	146.46c	16.25ab	topped
2006/2007	3	Mw 1	0.91abc	58.49a	7.36b	7.01a	0.58a	12.04ab	124.76b	16.22ab	none
2006/2007	A4	Mw 2	0.89ac	59.94a	8.09a	6.44a	0.25a	5.46a	96.27a	14.96b	topped
2006/2007	8	Mw 3	0.81c	48.96b	7.21b	6.72a	0.50a	8.81ab	132.82b	16.96a	none
2006/2007	A12	Ld 1	0.63d	40.38c	7.02b	6.83a	1.63b	18.21b	121.19b	15.58ab	none
2007/2008	A9	Hw 3	1.31b	69.14cd	8.21a	5.67c	1.67ab	24.75a	156.00a	17.64ab	none
2007/2008	5	Hw 4	1.22bc	74.81c	9.42ab	9.00ab	1.67ab	36.79a	113.00a	19.14ab	none
2007/2008	3	Hw 5	1.13ab	65.41ac	9.08b	9.33b	2.33a	57.92a	118.00a	18.33b	none
2007/2008	A3	Mw 4	1.09ac	60.39abd	7.47ab	6.33a	0.83b	19.88a	127.00a	17.68ab	none
2007/2008	8	Mw 5	1.04ac	58.95abd	7.75b	6.67ab	2.00a	47.96a	124.00a	16.86a	none
2007/2008	2	Mw 6	0.99a	57.67ab	8.40ab	8.33ab	1.50ab	54.63a	126.00a	17.83ab	none
2007/2008	A12	Ld 2	0.95a	51.31b	6.97a	8.33ab	2.00a	55.58a	132.50a	18.05ab	none

Table 3b Mean grapevine response data per plot (24 vines) for the 2006/2007 and 2007/2008 seasons.

Season	Plot	Plot code	Yield per vine (kg)	Fresh weight per berry (g)	Number of bunches per vine	Mass per bunch (g)	[X]PAR in bunch zone	[Y]PDWP for season (MPa)	Water [Z]CR/3 for season
2006/2007	1	Hw 1	4.71ab	1.60ab	26.58a	175.22a	**nm	-0.36b	0.52a
2006/2007	A2	Hw 2	4.34ab	1.61ab	26.15a	166.38a	nm	-0.22a	0.50ab
2006/2007	3	Mw 1	3.87a	1.51a	28.46a	135.69b	nm	-0.46c	0.52a
2006/2007	A4	Mw 2	4.31ab	1.65ab	26.75a	161.77a	nm	-0.16d	0.52a
2006/2007	8	Mw 3	5.22b	1.68b	28.54a	181.50a	nm	-0.25a	0.55c
2006/2007	A12	Ld 1	2.54c	1.53a	18.84b	130.96b	nm	-0.33a	0.48b
2007/2008	A9	Hw 3	3.99ab	1.47b	27.87a	141.22ab	0.006c	-0.15c	0.51a
2007/2008	5	Hw 4	4.02ab	1.44ab	25.87a	153.13a	0.017ac	-0.18c	0.50a
2007/2008	3	Hw 5	3.39a	1.34a	26.74a	124.45c	0.011ac	-0.30a	0.50a
2007/2008	A3	Mw 4	3.91ab	1.38ab	28.67a	137.21bcd	0.006c	-0.16c	0.47ab
2007/2008	8	Mw 5	4.10b	1.38ab	26.68a	152.67ad	0.010b	-0.20bc	0.51a
2007/2008	2	Mw 6	3.65ab	1.39ab	25.67a	142.67ab	0.013ab	-0.28ab	0.49a
2007/2008	A12	Ld 2	3.40a	1.50b	25.39a	131.78bc	0.008ac	-0.13c	0.41b

** Not measured (nm); [Y]Average of 24 shoots in 2006/2007 season and 12 shoots in 2007/2008 taking into account shoots without any lateral shoots. [X] Photosynthetic active radiation (PAR) measured post–veraison (29 Feb 08) expressed as a ratio of bunch zone PAR:ambient PAR; [Y]Predawn plant water potential (PDWP); [Z] Total neutron probe soil water count ratios of 3 soil depths (30 cm, 60cm, 90cm) divided by three.
Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1; Ld2)
Significant differences: indicated with abcd when not bearing the same letter indicating significant difference with $p \leq 0.05$ within each season between plots.

Table 4 Mean values of individual carotenoids and chlorophylls per berry fresh weight (fw) of four stages (pre-veraison, veraison, post-veraison, harvest) of ripening during the 2006/2007 ripening season.

Maturation Stage	Date	Code	Plot	Weight per berry without seed (g)	Zea	Lut	ep-β-car	β-car	*cis* β-car	Tot Car	Chl a	Chl b	Total Chl	Malic acid	Total (Glucose + Fructose)	Total Tannin	Total Anthocyanin
					**Average µg/berry fresh weight								**Average mg/berry fresh weight				
Pre-veraison	11-Jan-07	1	Hw 1	0.86a	0.15ab	1.75ab	0.04ab	0.97ab	0.13ab	3.21ab	23.51ab	6.88ab	31.82ab	21.33a	25.53b	1.34a	0.03ab
	11-Jan-07	A2	Hw 2	0.76ab	0.27b	2.30b	0.05ab	1.29b	0.16b	4.34b	30.83b	8.55b	42.45b	16.74ab	16.07a	1.04a	0.00b
	11-Jan-07	3	Mw 1	0.77a	0.19a	1.83ab	0.05a	0.82ab	0.13ab	3.62ab	19.09ab	6.59ab	35.02a	18.61ab	14.54a	1.04a	0.01ab
	11-Jan-07	A4	Mw 2	0.80a	0.14a	1.72ab	0.04a	0.99ab	0.10a	3.17ab	22.60ab	6.35ab	30.75a	20.07ab	21.20bc	1.25a	0.05ab
	11-Jan-07	8	Mw 3	0.82a	0.20ab	1.83ab	0.08b	1.02ab	0.13ab	3.38ab	23.39ab	5.58a	32.28a	17.28ab	24.24b	1.35a	0.06ab
	11-Jan-07	A12	Ld 1	0.66b	0.15a	1.28a	0.02a	0.76a	0.09a	2.55a	17.56a	5.25a	23.49a	14.53b	16.80ac	1.04a	0.20a
Veraison	26-Jan-07	1	Hw 1	1.34a	0.20ac	2.11a	0.03b	1.04a	0.12ab	3.77a	22.73a	7.70a	30.85a	3.33b	93.37ab	1.35ab	11.98a
	26-Jan-07	A2	Hw 2	1.09b	0.11b	1.51c	0.00d	0.76c	0.09c	2.68c	16.46c	5.26c	22.16c	3.41bc	82.99b	1.16ab	8.07a
	26-Jan-07	3	Mw 1	1.12b	0.15ab	1.93ab	0.06a	0.97ab	0.11abc	3.32ab	21.35ab	7.11a	28.71ab	5.00a	89.74ab	0.96a	9.81a
	26-Jan-07	A4	Mw 2	1.29a	0.12bc	2.06ab	0.04ab	1.07a	0.13b	3.51ab	23.02a	7.58a	30.78a	4.85ac	91.31ab	1.47b	10.35a
	26-Jan-07	8	Mw 3	1.37a	0.21a	2.53d	0.17c	1.26d	0.15d	4.62d	26.93d	10.07b	37.47d	5.15a	106.41a	1.30ab	10.57a
	26-Jan-07	A12	Ld 1	1.13b	0.17ab	1.77bc	0.04ab	0.85bc	0.11ac	3.00bc	19.37bc	6.70a	26.07bc	4.32ab	86.60ab	1.08ab	10.24a
Post-veraison	8-Feb-07	1	Hw 1	1.46a	0.14a	2.50c	0.49a	1.21a	0.11abc	4.57a	24.68a	2.65b	35.37a	2.44a	149.52a	2.40c	19.36a
	8-Feb-07	A2	Hw 2	1.42ab	0.12d	1.77bd	0.06d	0.78b	0.13c	4.77a	15.36b	12.09a	27.45bc	2.50a	132.67ab	2.14ac	16.46a
	8-Feb-07	3	Mw 1	1.38ab	0.09ab	2.05ab	0.41ab	1.01ab	0.10ab	3.73a	21.22ab	9.38a	30.60ab	2.27ab	130.08ab	1.59ab	18.76a
	8-Feb-07	A4	Mw 2	1.40ab	0.10ab	2.18ac	0.44ab	0.97ab	0.12bc	3.85a	23.28ac	2.34b	33.38ab	2.40ab	142.10ab	1.32b	19.75a
	8-Feb-07	8	Mw 3	1.51a	0.10b	2.09ad	0.32b	1.00ab	0.09ad	3.69a	21.07ab	3.51b	29.60ab	2.29ab	138.10ab	2.45c	18.53a
	8-Feb-07	A12	Ld 1	1.29b	0.10c	1.69b	0.19c	0.78b	0.08d	2.99a	16.61bc	4.89b	22.78c	1.58b	124.22b	1.98ac	17.86a
Harvest	7-Mar-07	1	Hw 1	1.50a	0.05	1.63a	0.21a	0.65a	0.06bc	2.71a	12.13a	2.89a	16.44a	1.25b	154.46ab	0.85ab	22.27a
	7-Mar-07	A2	Hw 2	1.50a	nd*	1.43a	0.27ab	0.58a	0.05a	2.43a	11.17a	2.16ab	15.50a	0.60a	142.57a	0.98ab	21.10a
	7-Mar-07	3	Mw 1	1.41a	nd	1.59a	0.29ab	0.66a	0.05a	2.70a	12.05a	2.29ab	16.47a	0.65a	139.86a	0.71a	21.34a
	7-Mar-07	A4	Mw 2	1.57a	nd	1.53a	0.39b	0.62a	0.05ac	2.65a	12.36a	1.57b	17.41a	1.06ab	135.92a	0.90ab	23.28a
	7-Mar-07	8	Mw 3	1.56a	nd	1.60a	0.29ab	0.63a	0.06b	2.64a	11.98a	2.38a	16.22a	1.04ab	172.48b	1.20b	21.03a
	7-Mar-07	A12	Ld 1	1.40a	nd	1.51a	0.24ab	0.58a	0.06ab	2.54a	10.76a	3.72c	14.75a	0.52a	147.32a	1.10b	21.47a

**Average (Ave) calculated from 4 biological replicates each analysed in triplicate

* Not detected (nd)

Carotenoids: Zeaxanthin (Zea); Lutein (Lut); 5,8-epoxy-β-carotene (ep-β-car); β-carotene (β-car); *cis* β-carotene (*cis* β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds (see chapter 3)

Chlorophyll: Chlorophyll a (Chl a); Chlorophyll b (Chl b); Total chlorophyll (Tot chl) calculated as the sum of all chlorophylls and detected chlorophyll derivatives (see chapter 3)

Significant differences: indicated with abcd when not bearing the same letter indicating significant difference with $p \leq 0.05$ between plots for specific maturation stage.

Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1; Ld2)

Table 5 Mean values of individual carotenoids and chlorophylls µg/g fresh weight (fw) of four stages (pre-veraison, veraison, post-veraison, harvest) of ripening during the 2006/2007 ripening season.

Maturation Stage	Date	Code	Plot	Weight per berry without seed (g)	Zea	Lut	ep-β-car	β-car	*cis* β-car	Total Car	Chl a	Chl b	Total Chl	Malic acid	Total (Glucose + Fructose)	Total Tannin	Total Anthocyanin
					**Average µg/g fresh weight									**Average mg/g fresh weight			
Pre-veraison	11-Jan-07	1	Hw 1	0.86a	0.17a	2.03a	0.04a	1.13a	0.14ab	3.71a	27.18a	7.96a	36.79a	24.73a	29.58a	1.56a	0.03a
	11-Jan-07	A2	Hw 2	0.76ab	0.36a	3.02a	0.07a	1.68ab	0.21c	5.70ab	40.24ab	11.31b	55.38b	21.84a	21.50bc	1.35a	0.00a
	11-Jan-07	3	Mw 1	0.77a	0.24a	2.38a	0.07ab	1.07b	0.17ac	4.70b	24.89b	8.53ab	45.41ab	24.09a	18.91b	1.33a	0.01a
	11-Jan-07	A4	Mw 2	0.80a	0.17a	2.16a	0.05a	1.24ab	0.12b	3.99ab	28.42a	7.97a	38.68a	25.19a	26.59ac	1.56a	0.05a
	11-Jan-07	8	Mw 3	0.82a	0.24a	2.24a	0.10b	1.25ab	0.16abc	4.12ab	28.59a	6.81a	39.46a	21.10a	29.66a	1.65a	0.06a
	11-Jan-07	A12	Ld 1	0.66b	0.22a	1.93a	0.03a	1.14ab	0.14ab	3.81ab	26.22a	7.86a	35.07a	21.65a	26.23ac	1.56a	0.20a
Veraison	26-Jan-07	1	Hw 1	1.34a	0.15a	1.57ab	0.02ab	0.77ab	0.09a	2.80ab	16.87ab	5.71ab	22.90ab	2.49a	69.62a	1.00a	8.93a
	26-Jan-07	A2	Hw 2	1.09b	0.10a	1.39b	0.00b	0.70b	0.08a	2.46b	15.08b	4.82b	20.30b	3.15ac	76.18a	1.06a	7.38a
	26-Jan-07	3	Mw 1	1.12b	0.13a	1.77a	0.05c	0.89a	0.10ab	3.04ac	19.45a	6.46ac	26.17ac	4.53b	81.46a	0.86a	8.92a
	26-Jan-07	A4	Mw 2	1.29a	0.10a	1.60ab	0.03a	0.83ab	0.10ab	2.73ab	17.85ab	5.88a	23.90abc	3.76ab	70.88a	1.13a	7.97a
	26-Jan-07	8	Mw 3	1.37a	0.15a	1.84a	0.13d	0.92a	0.11b	3.37c	19.64a	7.33c	27.33c	3.75ab	77.26a	0.94a	7.67a
	26-Jan-07	A12	Ld 1	1.13b	0.15a	1.58ab	0.03ac	0.76ab	0.09ab	2.67ab	17.26ab	5.97	23.23abc	3.87bc	77.12a	0.96a	9.12a
Post-veraison	8-Feb-07	1	Hw 1	1.46a	0.09a	1.71a	0.34a	0.83a	0.08ab	3.14a	16.95a	1.81a	24.30a	1.68ab	102.47a	1.65a	13.31a
	8-Feb-07	A2	Hw 2	1.42ab	0.08a	1.24b	0.04d	0.55b	0.09b	3.34a	10.85b	8.45a	19.30bc	1.75b	93.08a	1.50a	11.57a
	8-Feb-07	3	Mw 1	1.38ab	0.07a	1.49ab	0.30ab	0.73ab	0.07ac	2.71a	15.40ab	6.81a	22.21ab	1.61ab	94.07a	1.19ab	13.75a
	8-Feb-07	A4	Mw 2	1.40ab	0.07a	1.56ac	0.32a	0.70ab	0.09bc	2.76a	16.67a	1.70b	23.88ab	1.72ab	101.66a	0.95b	14.18a
	8-Feb-07	8	Mw 3	1.51a	0.07a	1.39bc	0.21bc	0.66ab	0.06a	2.45a	14.02ab	2.34b	19.69bc	1.52ab	91.91a	1.63a	12.31a
	8-Feb-07	A12	Ld 1	1.29b	0.08a	1.31bc	0.14c	0.61ab	0.06a	2.33a	12.91ab	3.76b	17.70c	1.22a	95.58a	1.54a	13.83a
Harvest	7-Mar-07	1	Hw 1	1.50a	0.03b	1.08a	0.14a	0.43a	0.04a	1.80a	8.01a	1.91a	10.87a	0.81a	103.01ab	0.57a	13.40a
	7-Mar-07	A2	Hw 2	1.50a	0.00a	0.96a	0.18a	0.39a	0.03b	1.62a	7.44a	1.44ab	10.33a	0.40b	94.96bc	0.65ab	13.52b
	7-Mar-07	3	Mw 1	1.41a	0.00a	1.14a	0.21a	0.47a	0.03ab	1.92a	8.57a	1.62a	11.71a	0.45b	100.02abc	0.52a	16.56b
	7-Mar-07	A4	Mw 2	1.57a	0.00a	0.99a	0.25a	0.40a	0.03b	1.71a	7.98a	1.02b	11.23a	0.67ab	86.87c	0.55a	13.13a
	7-Mar-07	8	Mw 3	1.56a	0.00a	1.02a	0.19a	0.41a	0.04ab	1.69a	7.68a	1.53a	10.39a	0.67ab	110.25a	0.75ab	13.72a
	7-Mar-07	A12	Ld 1	1.40a	0.00a	1.08a	0.17a	0.41a	0.04ab	1.81a	7.63a	2.64c	10.45a	0.36b	105.02ab	0.81b	15.42ab

**Average (Ave) calculated from 4 biological replicates which each analysed in triplicate; * Not detected (nd)
Significant differences: indicated with abcd when not bearing the same letter indicating significant difference with $p \leq 0.05$. Significant differences are only valid between plots for specific maturation stage.
Carotenoids: Zeaxanthin (Zea); Lutein (Lut); 5,8-epoxy-β-carotene (ep-β-car); β-carotene (β-car); *cis* β-carotene (*cis* β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds (see chapter 3)
Chlorophyll: Chlorophyll a (Chl a); Chlorophyll b (Chl b); Total chlorophyll (Tot chl) calculated as the sum of all chlorophylls and detected chlorophyll derivatives (see chapter 3)
Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1; Ld2)

Table 6 Mean values of individual carotenoids and chlorophylls per berry fresh weight (fw) of four (pre-veraison, veraison, post-veraison, harvest) stages of ripening during the 2007/2008 ripening season.

Maturation Stage	Date	Code	Plot	Weight per berry without seed (g)	Zea	Lut	ep-β-car	β-car	*cis* β-car	Tot Car	Chl a	Chl b	Tot Chl	Malic acid	Total (glucose + fructose)	Total Tannin	Tot Anthocyanin
				**Average µg/berry fresh weight										**Average mg/berry fresh weight			
Pre-veraison	10-Jan-08	A9	Hw 3	0.73ab	0.24abc	2.10b	0.24a	1.15ab	0.16bcd	4.40bc	19.13ab	7.68ab	27.80bc	15.01a	8.20c	0.91a	0.00a
	10-Jan-08	5	Hw 4	0.78b	0.33bc	1.95b	0.17a	1.10a	0.18ab	4.19b	14.39b	7.25ac	21.08b	19.94a	13.78b	0.92a	0.00a
	10-Jan-08	3	Hw 5	0.68a	0.09a	2.03b	0.15a	1.14ab	0.15bc	4.00bc	18.67bc	7.47ab	27.19bc	16.39a	8.87ac	0.87a	0.00a
	10-Jan-08	A3	Mw 3	0.75ab	0.13ab	1.94bc	0.16a	1.10a	0.16bcd	3.89bc	19.11ab	7.94bc	27.99bc	19.60a	13.76b	0.98a	0.00a
	10-Jan-08	8	Mw 4	0.72ab	0.40c	2.30ab	0.23a	1.27b	0.19ad	4.95ac	21.79ac	8.40b	31.43ac	19.90a	11.51abc	1.11a	0.00a
	10-Jan-08	2	Mw 5	0.75ab	0.16ab	2.58a	0.21a	1.45c	0.20a	5.21a	24.81a	9.75d	36.05a	20.22a	12.46ab	0.82a	0.00a
	10-Jan-08	A12	Ld 2	0.77b	0.28abc	1.53c	0.15a	1.01a	0.14c	3.48bc	16.91bc	6.87	24.25bc	17.77a	9.47ac	0.97a	0.00a
Post-veraison	31-Jan-08	A9	Hw 3	1.32b	0.14a	3.45b	3.47a	1.58b	0.22b	9.88b	27.96b	13.52c	42.55b	9.44b	124.10a	1.44a	12.11c
	31-Jan-08	5	Hw 4	1.23ab	0.11a	2.30a	1.25a	1.07a	0.14a	5.69a	17.69a	7.64a	26.27a	7.90ab	105.60a	1.10ab	12.35ac
	31-Jan-08	3	Hw 5	1.20ab	0.14a	2.70ab	1.46a	1.26ab	0.17ab	6.38a	20.23a	7.54a	29.64a	7.00a	127.23a	1.37a	14.85ab
	31-Jan-08	A3	Mw 3	1.27ab	0.11a	2.58ab	2.21a	1.11a	0.16a	6.87a	19.46a	8.93ab	28.90a	7.33ab	134.46a	1.46a	15.00b
	31-Jan-08	8	Mw 4	1.28ab	0.12a	2.90ab	2.52a	1.27ab	0.16a	7.75ab	21.36ab	9.87ab	32.02ab	5.97a	124.80a	0.65b	13.09abc
	31-Jan-08	2	Mw 5	1.17a	0.14a	2.72ab	2.10a	1.15a	0.17ab	7.08a	20.15a	8.95ab	30.14a	7.27ab	129.52a	1.44a	14.20abc
	31-Jan-08	A12	Ld 2	1.27ab	0.12a	3.21ab	3.00a	1.46ab	0.19ab	8.86ab	25.04ab	11.4bc	37.52ab	7.11ab	115.17a	0.94b	12.47ac
Post-veraison	21-Feb-08	A9	Hw 3	1.47c	0.13a	2.39a	1.37bcd	0.99b	0.13bcd	5.58a	14.83a	6.13a	21.68a	4.89bc	175.60b	0.82a	22.44a
	21-Feb-08	5	Hw 4	1.33b	0.09a	2.41a	2.42ab	0.96ab	0.12ab	6.67a	15.00a	6.80a	23.67a	5.14b	169.03ab	0.78a	22.47a
	21-Feb-08	3	Hw 5	1.26ab	0.08a	1.99a	1.19a	0.77a	0.10a	4.55a	11.86a	5.12a	17.82a	3.11a	137.13a	1.51b	20.74a
	21-Feb-08	A3	Mw 3	1.30ab	0.08a	3.44b	7.16cd	1.21c	0.15cd	13.73b	19.71b	10.37b	30.29b	4.77ab	158.04ab	0.90a	22.08a
	21-Feb-08	8	Mw 4	1.30ab	0.07a	2.62a	1.75abc	1.00ab	0.13abc	6.56a	15.51a	6.97a	23.52a	3.65ac	153.83ab	1.56bc	18.90a
	21-Feb-08	2	Mw5	1.24a	0.09a	1.93a	2.78ab	0.72a	0.10ab	6.27a	10.87a	5.14a	16.10a	4.22ab	141.63a	0.85a	20.62a
	21-Feb-08	A12	Ld 2	1.34b	0.12a	3.40b	5.97d	1.38c	0.17d	12.40b	21.62b	10.39b	33.54b	3.96ab	125.08a	2.00c	19.99a
Harvest	3-Mar-08	A9	Hw 3	1.39b	0.03a	1.75a	0.85a	0.73b	0.09a	3.91b	10.32b	3.84ab	15.22b	2.79ab	167.86b	0.52a	15.83a
	3-Mar-08	5	Hw 4	1.37ab	0.05a	1.65a	1.19a	0.57ab	0.07a	3.95ab	9.08ab	4.32ac	13.52ab	2.32a	169.72b	0.53a	18.28a
	3-Mar-08	3	Hw 5	1.24a	0.00b	1.97a	1.90a	0.69ab	0.08a	5.21ab	9.85ab	4.59a	14.58ab	3.17ab	155.40ab	0.75ab	20.95a
	3-Mar-08	A3	Mw 3	1.30ab	0.00b	1.74a	1.84a	0.65ab	0.08a	4.86ab	8.59ab	3.28bc	12.51ab	2.01a	126.62ab	0.54a	15.50a
	3-Mar-08	8	Mw 4	1.25a	0.07c	1.63a	1.60a	0.59ab	0.08a	4.51ab	8.22ab	3.00b	11.89ab	3.73b	159.52ab	0.94b	21.53a
	3-Mar-08	2	Mw 5	1.32ab	0.03a	1.53a	1.85a	0.52a	0.07a	4.52a	7.30a	3.40ab	10.80a	2.13a	110.40a	0.73ab	17.86a
	3-Mar-08	A12	Ld 2	1.42b	0.04a	1.73a	1.63a	0.63ab	0.07a	4.48ab	9.74ab	4.49ac	14.33ab	3.03ab	137.10ab	0.90ab	19.01a

**Average (Ave) calculated from 4 biological replicates each analysed in triplicate

Carotenoids: Zeaxanthin (Zea); Lutein (Lut); 5,8-epoxy-β-carotene (ep-β-car); β-carotene (β-car); *cis* β-carotene (*cis* β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds (see chapter 3)

Chlorophyll: Clorophyll a (Chl a); Chlorophyll b (Chl b); Total chlorophyll (Tot chl) calculated as the sum of all chlorophylls and detected chlorophyll derivatives (see chapter 3)

Significant differences: indicated with abcd when not bearing the same letter indicating significant difference with $p \leq 0.05$ between plots for specific maturation stage.

Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1; Ld2)

Table 7 Mean values of individual carotenoids and chlorophylls µg/g fresh weight (fw) of four (pre-veraison, veraison, post-veraison, harvest) stages of ripening during the 2007/2008 ripening season.

Maturation Stage	Date	Code	Plot	Weight per berry without seed (g)	Zea	Lut	ep-β-car	β-car	*cis* β-car	Tot Car	Chl a	Chl b	Total Chl	Malic acid	Total Glucose + Fructose)	Total Tannin	Total Athocyanin
					**Average µg/g fresh weight									**Average mg/g fresh weight			
Pre-veraison	10-Jan-08	A9	Hw 3	0.73ab	0.32abc	2.87bc	0.33b	1.58bce	0.22bc	6.02ab	26.20ab	10.53ac	38.10bd	20.51a	11.28c	1.24ab	0.00a
	10-Jan-08	5	Hw 4	0.78b	0.43bc	2.50b	0.21ab	1.41de	0.23abd	4.04b	18.12c	9.31bc	26.53c	25.53a	17.62b	1.18ab	0.00a
	10-Jan-08	3	Hw 5	0.68a	0.13a	2.99ab	0.21ab	1.68bc	0.22bc	5.89ab	27.47ab	10.99a	40.00ab	24.13a	12.94ac	1.27ab	0.00a
	10-Jan-08	A3	Mw 4	0.75ab	0.17ab	2.61b	0.21a	1.48cd	0.21cd	4.10b	25.74bd	10.69a	37.40bd	26.23a	18.31b	1.30ab	0.00a
	10-Jan-08	8	Mw 5	0.72ab	0.54c	3.17ac	0.31ab	1.76ab	0.26ab	6.80a	30.04ad	11.57a	43.32ad	27.38a	15.90b	1.50b	0.00a
	10-Jan-08	2	Mw 6	0.75ab	0.20ab	3.44a	0.28ab	1.93a	0.26a	6.94a	33.09a	12.99d	48.07a	26.97a	16.43ab	1.10a	0.00a
	10-Jan-08	A12	Ld 2	0.77b	0.36abc	1.97d	0.20a	1.30d	0.18c	4.50b	21.90bc	8.91b	31.41bc	23.11a	12.16ac	1.27ab	0.00a
Post-veraison	31-Jan-08	A9	Hw 3	1.32b	0.11a	2.64a	2.70a	1.21a	0.17b	7.60a	21.40b	10.33b	32.56b	7.17a	94.43ab	1.09a	9.24b
	31-Jan-08	5	Hw 4	1.23ab	0.09a	1.87a	1.00a	0.87a	0.11a	4.60a	14.36a	6.20ac	21.33a	6.47a	86.00b	0.90ab	10.05bc
	31-Jan-08	3	Hw 5	1.20ab	0.12a	2.25a	1.22a	1.05a	0.14ab	5.33a	16.85ab	6.28a	24.70ab	5.81ab	105.69ab	1.14aa	12.36a
	31-Jan-08	A3	Mw 4	1.27ab	0.08a	2.04a	1.76a	0.88a	0.13ab	5.44a	15.36a	7.06ac	22.81a	5.74ab	105.38ab	1.14a	11.74ac
	31-Jan-08	8	Mw 5	1.28ab	0.09a	2.28a	2.01a	1.00a	0.13ab	6.11a	16.75ab	7.76ab	25.11ab	4.64b	96.75ab	0.50c	10.24bc
	31-Jan-08	2	Mw 6	1.17a	0.12a	2.35a	1.83a	0.99a	0.15ab	6.12a	17.36ab	7.71ab	25.98ab	6.22ab	110.61a	1.23a	12.17a
	31-Jan-08	A12	Ld 2	1.27ab	0.10a	2.53a	2.36a	1.15a	0.15ab	6.97a	19.69ab	8.97bc	29.49ab	5.57ab	90.62ab	0.74bc	9.86b
Post-veraison	21-Feb-08	A9	Hw 3	1.47c	0.08a	1.60a	0.91ab	0.66a	0.09a	3.73a	9.90a	4.17a	14.48a	3.23ab	117.10a	0.54a	14.91a
	21-Feb-08	5	Hw 4	1.33b	0.07a	1.80a	1.81ab	0.72a	0.09a	5.00a	11.23a	5.09a	17.75a	3.86b	126.77a	0.59a	16.90a
	21-Feb-08	3	Hw 5	1.26ab	0.06a	1.56a	0.93b	0.61a	0.07a	3.57a	9.30a	4.05a	13.98a	2.44a	107.55a	1.19b	16.31a
	21-Feb-08	A3	Mw 4	1.30ab	0.06a	2.65b	5.51d	0.93b	0.11b	10.56b	15.16b	7.98b	23.30b	3.65ab	121.60a	0.69a	16.97a
	21-Feb-08	8	Mw 5	1.30ab	0.05a	2.03a	1.35ab	0.77a	0.10a	5.07a	12.01a	5.36a	18.21a	2.83ab	119.35a	1.21bc	14.65a
	21-Feb-08	2	Mw 6	1.24a	0.08a	1.57a	2.30a	0.59a	0.08a	5.14a	8.86a	4.19a	13.13a	3.43ab	114.61a	0.69a	16.74a
	21-Feb-08	A12	Ld 2	1.34b	0.09a	2.49b	4.38c	1.01b	0.13b	9.10b	15.86b	7.75b	24.60b	2.90ab	91.50a	1.47c	14.65a
Harvest	3-Mar-08	A9	Hw 3	1.39b	0.02a	1.28a	0.62a	0.53a	0.07a	2.85a	7.53a	2.80ab	11.10a	2.02ab	121.51ab	0.38a	11.56b
	3-Mar-08	5	Hw 4	1.37ab	0.03a	1.22a	0.91a	0.42a	0.05a	2.96a	6.71a	3.20ab	9.99a	1.70ab	124.91ab	0.39a	13.47ab
	3-Mar-08	3	Hw 5	1.24a	0.00b	1.60a	1.55a	0.56a	0.07a	4.23a	7.98a	3.72b	11.81a	2.56bc	125.84bc	0.60ab	16.98a
	3-Mar-08	A3	Mw 4	1.30ab	0.00b	1.34a	1.42a	0.50a	0.06a	3.75a	6.62a	2.53a	9.65a	1.52a	96.65a	0.41a	11.82ab
	3-Mar-08	8	Mw 5	1.25a	0.06c	1.30a	1.28a	0.47a	0.06a	3.60a	6.56a	2.40a	9.49a	2.98c	127.33c	0.75b	17.17a
	3-Mar-08	2	Mw 6	1.32ab	0.02a	1.19a	1.49a	0.40a	0.05a	3.57a	5.66a	2.63a	8.36a	1.63a	86.40a	0.58ab	13.89ab
	3-Mar-08	A12	Ld 2	1.42b	0.02a	1.22a	1.16a	0.44a	0.05a	3.17a	6.88a	3.17ab	10.12a	2.14abc	96.68abc	0.64ab	13.35ab

**Average (Ave) calculated from 4 biological replicates each analysed in triplicate

Carotenoids: Zeaxanthin (Zea); Lutein (Lut); 5,8-epoxy-β-carotene (ep-β-car); β-carotene (β-car); *cis* β-carotene (*cis* β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds (see chapter 3)

Chlorophyll: Clorophyll a (Chl a); Chlorophyll b (Chl b); Total chlorophyll (Tot chl) calculated as the sum of all chlorophylls and detected chlorophyll derivatives (see chapter 3)

Significant differences: indicated with abcd when not bearing the same letter indicating significant difference with $p \leq 0.05$ between plots for specific maturation stage.

Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1; Ld2)

Table 8 Volume, weight and ripening measurements for the 2006/2007 season per plot from veraison to harvest measured with different instruments.

Ripening Stage	Date	Code	Plot	Weight per 100 berries (g)	Volume per 100 berries (ml)	ºB (refracto-meter)	pH (electrode)	pH (metrhom)	Total Acid (metrhom) (g/l) WS*	Glucose + Fructose (g/l) WS	Brix WS	Total Acid (g/l) WS	pH WS	Tartaric acid (g/l) WS	Malic acid (g/l) WS	Folin C index WS	Anthocyanins (g/l) WS
Veraison	26-Jan-07	1	Hw 1	139.44	140	17.70	3.04	3.17	11.59	167.00	17.90	6.93	3.13	8.75	4.30	283.30	53.00
	26-Jan-07	A2	Hw 2	124.20	120	17.20	3.00	3.14	12.52	160.00	17.25	7.49	3.06	9.15	4.75	268.35	82.50
	26-Jan-07	3	Mw 1	118.40	119	17.00	2.99	3.13	13.11	153.50	16.80	7.78	3.08	9.60	4.65	356.30	75.50
	26-Jan-07	A4	Mw 2	125.44	130	16.40	3.01	3.16	12.19	151.50	16.50	7.29	3.10	8.55	4.80	293.90	76.50
	26-Jan-07	8	Mw 3	136.32	130	17.00	3.01	3.15	11.82	159.00	17.10	7.00	3.09	8.50	4.50	283.85	82.50
	26-Jan-07	A12	Ld 1	114.40	110	17.60	3.00	3.15	11.37	163.50	17.55	6.68	3.07	8.45	3.80	278.20	82.00
Post-veraison	8-Feb-07	1	Hw 1	150.80	140	20.90	3.21	3.41	7.91	187.00	19.70	5.27	3.27	8.00	2.60	288.90	48.00
	8-Feb-07	A2	Hw 2	164.33	150	20.80	3.17	3.39	8.11	176.00	18.70	5.78	3.18	8.40	3.00	231.10	68.00
	8-Feb-07	3	Mw 1	142.09	135	19.90	3.23	3.43	7.83	175.00	18.60	5.51	3.21	8.40	2.70	286.60	70.00
	8-Feb-07	A4	Mw 2	152.93	140	19.80	3.21	3.42	7.62	176.00	18.70	6.00	3.18	8.20	3.40	252.60	44.00
	8-Feb-07	8	Mw 3	157.18	148	18.60	3.18	3.39	7.79	180.00	18.90	5.38	3.21	7.80	2.90	238.50	40.00
	8-Feb-07	A12	Ld 1	129.31	120	17.10	3.18	3.37	7.66	187.00	19.60	5.18	3.20	7.90	2.30	227.90	54.00
Post-veraison	15-Feb-07	1	Hw 1	185.14	178	20.00	3.19	3.42	7.48	212.00	21.70	4.32	3.38	8.00	1.50	183.40	20.00
	15-Feb-07	A2	Hw 2	172.98	168	17.90	3.18	3.41	7.5	203.00	20.90	4.35	3.37	7.80	1.70	233.50	32.00
	15-Feb-07	3	Mw 1	153.24	150	20.20	3.90	3.43	7.5	204.00	21.10	4.26	3.41	8.10	1.50	241.30	7.00
	15-Feb-07	A4	Mw 2	148.43	140	21.00	3.13	3.36	7.83	207.00	21.30	4.53	3.34	7.90	1.60	212.00	33.00
	15-Feb-07	8	Mw 3	185.04	180	19.70	3.19	3.42	7.29	209.00	21.30	4.26	3.37	7.70	1.60	189.40	36.00
	15-Feb-07	A12	Ld 1	147.10	140	20.70	3.15	3.38	7.24	208.00	21.30	4.38	3.36	8.10	1.40	238.40	21.00
Post-veraison	23-Feb-07	1	Hw 1	166.78	160	22.90	3.23	3.54	6.28	234.00	23.40	3.97	3.51	8.20	1.20	207.30	76.00
	23-Feb-07	A2	Hw 2	157.60	150	20.00	3.23	3.54	6.1	233.00	23.30	3.90	3.51	8.10	1.40	203.40	85.00
	23-Feb-07	3	Mw 1	146.36	140	20.10	3.24	3.55	6.09	228.00	22.90	3.91	3.55	8.10	1.20	273.30	88.00
	23-Feb-07	A4	Mw 2	159.50	150	21.00	3.24	3.55	6.08	234.00	23.30	3.75	3.51	7.70	1.40	240.80	91.00
	23-Feb-07	8	Mw 3	176.98	165	21.70	3.22	3.51	6.1	231.00	23.00	3.90	3.47	8.00	1.50	206.40	80.00
	23-Feb-07	A12	Ld 1	146.03	145	20.80	3.17	3.47	6.22	234.00	23.40	3.96	3.45	8.00	1.10	268.20	91.00
Post-veraison	2-Mar-07	1	Hw 1	156.69	150	24.00	**nm	3.61	6.06	240.00	23.70	3.54	3.64	7.70	0.90	261.90	82.00
	2-Mar-07	A2	Hw 2	157.31	150	23.30	nm	3.56	6.4	238.00	23.50	3.76	3.57	8.00	1.20	237.30	83.00
	2-Mar-07	3	Mw 1	141.60	130	23.40	nm	3.61	6.25	248.00	24.40	3.71	3.69	8.30	0.80	305.70	74.00
	2-Mar-07	A4	Mw 2	155.07	150	23.30	nm	3.65	5.78	243.00	23.90	3.54	3.71	7.90	1.10	277.20	96.00
	2-Mar-07	8	Mw 3	152.65	145	23.90	nm	3.59	6.09	228.00	22.60	3.34	3.58	7.40	0.90	220.60	85.00
	2-Mar-07	A12	Ld 1	151.52	140	24.00	nm	3.56	6.03	250.00	24.50	3.63	3.59	8.00	0.80	297.50	73.00
Harvest	7-Mar-07	1	Hw 1	170.67	162	24.10	3.24	3.60	6.61	248.00	24.70	3.80	3.59	8.10	0.90	259.00	80.00
	7-Mar-07	A2	Hw 2	170.10	160	23.50	3.18	3.54	6.83	237.00	23.70	3.84	3.52	7.90	0.90	213.90	72.00
	7-Mar-07	3	Mw 1	155.07	149	24.10	3.24	3.60	6.66	245.00	24.30	3.21	3.60	6.70	0.40	294.40	61.00
	7-Mar-07	A4	Mw 2	180.99	170	23.50	3.16	3.53	6.51	242.00	24.00	3.51	3.44	6.80	0.90	230.20	76.00
	7-Mar-07	8	Mw 3	180.18	170	23.60	3.29	3.64	6.13	241.00	24.10	3.51	3.62	7.40	0.90	272.30	76.00
	7-Mar-07	A12	Ld 1	162.83	158	24.40	3.23	3.59	6.31	250.00	24.90	3.57	3.59	7.80	0.70	278.90	76.00

*Measured with Wine scan (WS); ** Not measured (nm)

Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1-Ld2)

Table 9 Volume, weight and ripening measurements for the 2007/2008 season per plot from veraison to harvest measured with different instruments.

Ripening Stage	Date	Code	Plot	Weight per 100 berries (g)	Volume per 100 berries (ml)	°B (refracto-meter	pH (electrode)	pH (metrhom)	Total Acid (g/l)	Glucose + Fructose *WS	Brix WS	Total Acid WS	pH WS	Tartaric Acid WS	Malic Acid WS	Folin C index WS	Anthocyanins WS
Veraison	24-Jan-08	A9	Hw 3	128.38	120	14.7	2.74	2.96	14.42	146.00	15.40	9.40	3.00	8.90	7.10	316.20	90.00
	24-Jan-08	5	Hw 4	128.30	119	15.9	2.90	2.91	14.13	155.00	16.20	8.66	3.05	8.60	6.20	328.60	80.00
	24-Jan-08	3	Hw 5	121.66	113	16.5	2.94	2.86	13.82	161.00	16.70	8.50	3.01	8.80	5.70	306.10	77.00
	24-Jan-08	A3	Mw 3	137.68	122	15.9	2.96	2.88	13.2	156.00	16.20	8.20	3.03	8.10	5.70	276.80	75.00
	24-Jan-08	2	Mw 5	128.38	120	14.6	2.94	2.87	13.3	157.00	16.20	8.46	3.01	8.80	5.50	315.20	78.00
	24-Jan-08	8	Mw 4	128.90	120	14.8	2.91	2.83	13.2	147.00	15.40	8.14	3.00	8.30	5.40	282.70	100.00
	24-Jan-08	A12	Ld 2	127.51	118	15.1	2.88	2.82	15.37	147.00	15.50	9.70	2.94	9.10	7.00	302.80	80.00
Post-veraison	31-Jan-08	A9	Hw 3	135.89	130	18.4	3.08	3.19	11.2	178.00	18.30	6.81	3.21	8.00	4.00	295.00	58.00
	31-Jan-08	5	Hw 4	141.50	136	18.9	3.08	3.49	11.62	185.00	19.00	6.72	3.19	8.20	3.90	240.20	57.00
	31-Jan-08	3	Hw 5	131.30	125	19.5	3.07	3.23	10.46	194.00	19.60	6.25	3.20	8.40	2.80	255.10	63.00
	31-Jan-08	A3	Mw 3	140.90	150	20	3.10	3.29	10.12	195.00	19.70	6.06	3.21	7.70	3.20	220.30	64.00
	31-Jan-08	2	Mw 5	134.40	128	19.6	3.08	3.37	10.51	193.00	19.50	6.20	3.20	8.30	2.90	232.90	47.00
	31-Jan-08	8	Mw 4	138.70	132	18.6	6.06	3.27	10.6	182.00	18.60	6.06	3.18	7.80	2.90	231.10	74.00
	31-Jan-08	A12	Ld 2	143.30	137	18.4	3.03	3.21	11.1	179.00	18.30	6.65	3.15	8.10	3.50	224.50	63.00
Post-veraison	7-Feb-08	A9	Hw 3	138.26	121	21	3.19	3.39	8.97	208.00	21.00	5.38	3.35	7.30	2.60	294.40	79.00
	7-Feb-08	5	Hw 4	139.90	128	21.6	3.19	3.39	8.94	212.00	21.30	5.32	3.37	7.30	2.50	267.60	37.00
	7-Feb-08	3	Hw 5	131.12	125	22.7	3.20	3.40	8.39	224.00	22.40	5.04	3.35	7.60	1.80	236.80	48.00
	7-Feb-08	A3	Mw 3	132.38	121	22.6	3.20	3.46	8.56	222.00	22.30	5.06	3.36	7.50	1.90	216.00	48.00
	7-Feb-08	2	Mw 5	137.22	128	22	3.20	3.39	8.19	216.00	21.60	4.78	3.34	7.40	1.60	217.90	56.00
	7-Feb-08	8	Mw 4	137.22	130	20.8	3.14	3.33	8.59	202.00	20.40	5.23	3.31	7.60	2.10	248.10	68.00
	7-Feb-08	A12	Ld 2	142.64	130	21.2	3.16	3.36	8.94	209.00	21.00	5.40	3.33	7.70	2.40	306.10	81.00
Post-veraison	14-Feb-08	A9	Hw 3	143.95	130	21.9	3.30	3.47	7.92	217.00	21.70	4.44	3.44	7.10	1.60	263.30	89.00
	14-Feb-08	5	Hw 4	152.79	135	22.3	3.33	3.49	7.5	222.00	22.20	4.21	3.45	6.80	1.40	239.30	79.00
	14-Feb-08	3	Hw 5	134.82	120	23.1	3.32	3.48	7.29	230.00	23.00	4.15	3.48	7.40	0.90	217.00	80.00
	14-Feb-08	A3	Mw 3	157.51	140	22.8	3.32	3.50	7.39	226.00	22.60	4.15	3.48	7.00	1.10	250.70	72.00
	14-Feb-08	2	Mw 5	138.52	128	22.5	3.29	3.53	7.45	228.00	22.80	4.17	3.43	7.30	0.90	248.60	120.00
	14-Feb-08	8	Mw 4	147.50	135	21.9	3.28	3.45	7.34	214.00	21.50	4.10	3.40	7.10	1.00	243.80	114.00
	14-Feb-08	A12	Ld 2	155.81	142	22	3.29	3.45	7.65	218.00	21.90	4.28	3.42	7.00	1.40	227.80	89.00

Table 9 (continued) Volume, weight and ripening measurements for the 2007/2008 season per plot from veraison to harvest measured with different instruments.

Ripening Stage	Date	Code	Plot	Weight per 100 berries (g)	Volume per 100 berries (ml)	°B (refracto-meter	pH (electrode)	pH (metrhom)	Total Acid (g/l)	Glucose + Fructose *WS	Brix WS	Total Acid WS	pH WS	Tartaric Acid WS	Malic Acid WS	Folin C index WS	Anthocyanins WS
Post-veraison	21-Feb-08	A9	Hw 3	141.55	135	23.1	3.35	3.51	6.77	233.00	23.20	4.30	3.59	7.30	1.70	242.10	86.00
	21-Feb-08	5	Hw 4	141.52	130	23.5	3.40	3.54	6.23	237.00	23.70	4.01	3.58	7.00	1.10	223.40	73.00
	21-Feb-08	3	Hw 5	132.29	125	24.4	3.40	3.54	6.36	246.00	24.40	3.99	3.64	7.50	0.90	217.40	113.00
	21-Feb-08	A3	Mw 3	130.51	123	24.2	3.40	3.53	6.38	246.00	24.40	3.87	3.61	7.20	0.90	221.40	95.00
	21-Feb-08	2	Mw 5	141.83	135	23.1	3.35	3.52	6.41	239.00	23.80	3.92	3.58	7.40	0.70	270.10	86.00
	21-Feb-08	8	Mw 4	141.09	135	23.5	3.34	3.48	6.35	237.00	23.60	3.97	3.54	7.20	0.90	216.30	93.00
	21-Feb-08	A12	Ld 2	143.68	138	23.3	3.31	3.45	6.86	238.00	23.70	4.38	3.54	7.70	1.20	211.60	89.00
Harvest	3-Mar-08	A9	Hw 3	139.84	129	23.5	3.45	3.56	5.92	233.00	23.30	3.62	3.66	6.50	0.80	211.20	105.00
	3-Mar-08	5	Hw 4	142.65	128	23.3	3.45	3.57	5.76	229.00	23.00	3.48	3.64	6.60	0.60	242.50	92.00
	3-Mar-08	3	Hw 5	131.65	120	24.3	3.48	3.63	5.58	242.00	24.20	3.39	3.70	6.80	0.30	251.60	94.00
	3-Mar-08	A3	Mw 3	142.78	125	24	3.45	3.56	5.66	238.00	23.80	3.44	3.65	6.60	0.50	225.20	91.00
	3-Mar-08	2	Mw 5	129.87	118	24.1	3.44	3.56	5.66	240.00	24.00	3.33	3.61	6.80	0.20	193.00	102.00
	3-Mar-08	8	Mw 4	136.69	122	23.8	3.41	3.52	5.49	231.00	23.70	3.29	3.54	6.40	0.20	288.00	84.00
	3-Mar-08	A12	Ld 2	137.96	122	23.5	3.40	3.51	5.86	230.00	23.50	3.92	3.77	5.80	1.10	489.90	448.00

*Measured with Wine scan (WS)
Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld1-Ld2)

Table 10 Correlations of individual and total carotenoids and chlorophylls with ripeness measurements during the 2006/2007 ripening season.

	Zea µg/ berry fw	**Lut µg/ berry fw**	ep- **β-car µg/ berry fw**	**β-car µg/ berry fw**	**cis β-car µg/ berry fw**	**Total car µg/ berry fw**	**Chl a µg/ berry fw**	**Chl b µg/ berry fw**	**Total chl µg/ berry fw**
Malic acid mg/berry fw	.5533	-.0558	-.5122	.2188	.4882	.0400	.3537	.2921	.4920
	p=.000	p=.594	p=.000	p=.034	p=.000	p=.702	p=.000	p=.004	p=.000
Tot (gluc+fruc) mg/berry fw	-.6123	.1568	.6790	-.1567	-.4687	.0449	-.2936	-.3458	-.4177
	p=.000	p=.131	p=.000	p=.132	p=.000	p=.668	p=.004	p=.001	p=.000
Total tan mg/berry fw	.2224	.4673	.2175	.3507	.3212	.5147	.3084	.2292	.3835
	p=.031	p=.000	p=.035	p=.001	p=.002	p=.000	p=.002	p=.026	p=.000
Total anth mg/berry fw	-.6737	.0837	.7076	-.2174	-.5363	-.0165	-.3522	-.4012	-.4776
	p=.000	p=.422	p=.000	p=.035	p=.000	p=.875	p=.000	p=.000	p=.000

Carotenoids: Zeaxanthin (Zea); Lutein (Lut); 5,8-epoxy-β-carotene (ep-β-car); β-carotene (β-car); *cis* β-carotene (*cis* β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds (see chapter 3)
Chlorophyll: Chlorophyll a (Chl a); Chlorophyll b (Chl b); Total chlorophyll (Tot chl) calculated as the sum of all chlorophylls and detected chlorophyll derivatives (see chapter 3)
Ripening parameters: Malic acid; Total glucose and fructose (Tot (gluc+fruc); Total tannin (Tot tan); Total antocyanin (Tot anth)

Table 11 Correlations of individual and total carotenoids and chlorophylls with ripeness measurements during the 2007/2008 ripening season.

	Zea µg/ berry fw	**Lut µg/ berry fw**	ep- **β-car µg/ berry fw**	**β-car µg/ berry fw**	**cis β-car µg/ berry fw**	**Total car µg/ berry fw**	**Chl a µg/ berry fw**	**Chl b µg/ berry fw**	**Total chl µg/ berry fw**
Malic acid mg/berry fw	.5663	.0043	-.4223	.4531	.5307	-.2160	.4535	.3394	.4193
	p=.000	p=.965	p=.000	p=.000	p=.000	p=.023	p=.000	p=.000	p=.000
Tot (gluc+fruc) mg/berry fw	-.5353	.1263	.4016	-.3180	-.3982	.2483	-.3216	-.2233	-.2879
	p=.000	p=.189	p=.000	p=.001	p=.000	p=.009	p=.001	p=.019	p=.002
Total tan mg/berry fw	.1573	.3551	.1765	.3000	.2694	.2770	.2907	.3305	.3046
	p=.101	p=.000	p=.065	p=.001	p=.004	p=.003	p=.002	p=.000	p=.001
Total anth mg/berry fw	-.5532	.1290	.4999	-.3519	-.4561	.3203	-.3667	-.2471	-.3303
	p=.000	p=.179	p=.000	p=.000	p=.000	p=.001	p=.000	p=.009	p=.000

Carotenoids: Zeaxanthin (Zea); Lutein (Lut); 5,8-epoxy-β-carotene (ep-β-car); β-carotene (β-car); *cis* β-carotene (*cis* β-car); Total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds (see chapter 3)
Chlorophyll: Chlorophyll a (Chl a); Chlorophyll b (Chl b); Total chlorophyll (Tot chl) calculated as the sum of all chlorophylls and detected chlorophyll derivatives (see chapter 3)
Ripening parameters: Malic acid; Total glucose and fructose (Tot (gluc+fruc); Total tannin (Tot tan); Total antocyanin (Tot anth)

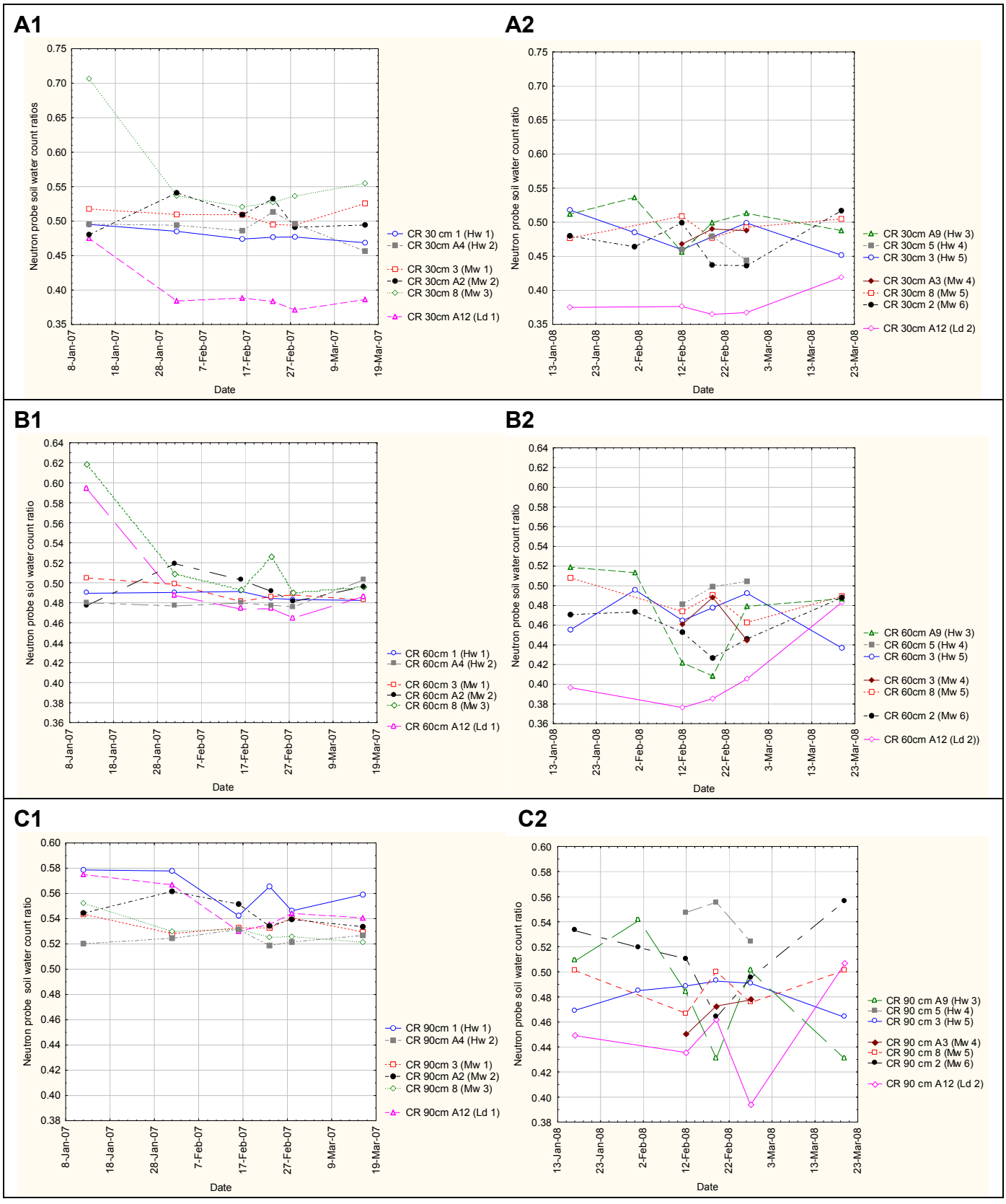
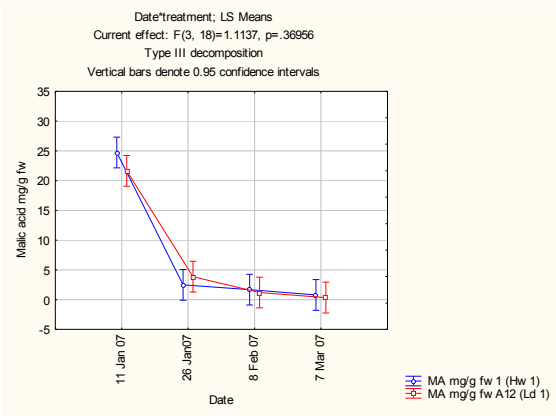
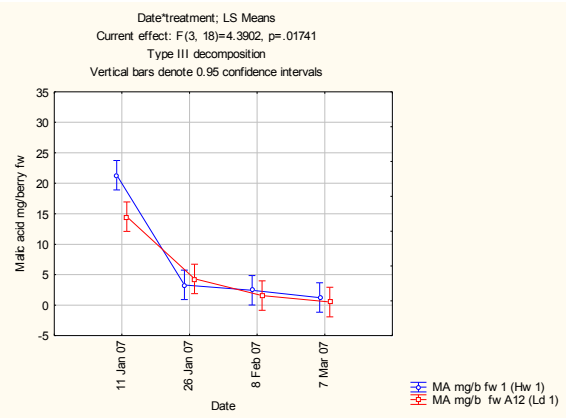


Figure 1 Neutron probe soil water count ratios of all the experimental plots, for the 1. 2006/2007 and 2.2007/2008 seasons respectively, measured at three depths respectively A. 30cm B. 60cm and C. 90cm. Plots classified as high vigour with high soil moisture (Hw 1-5), plots classified as medium vigour plots with high soil moisture (Mw 1-5), plots classified as low vigour with low soil moisture (Ld 1-2).

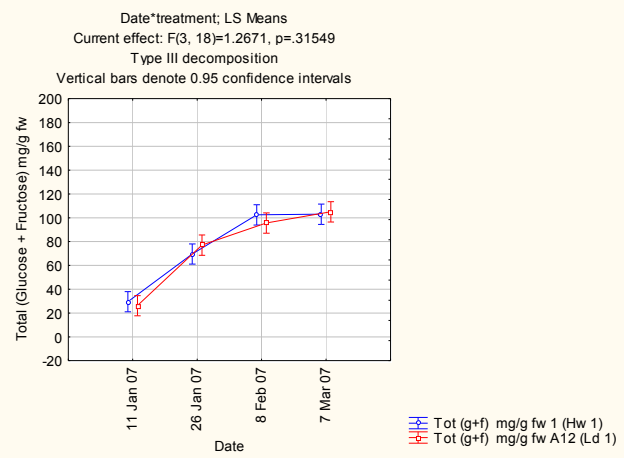
A1



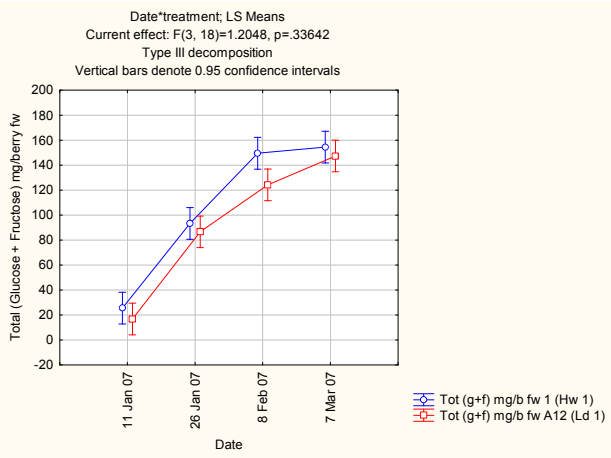
A2



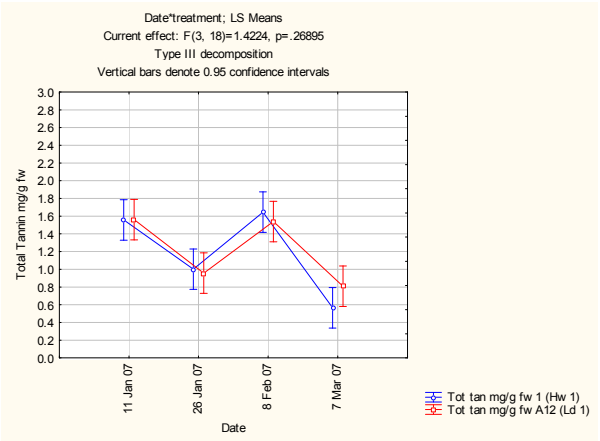
B1



B2



C1



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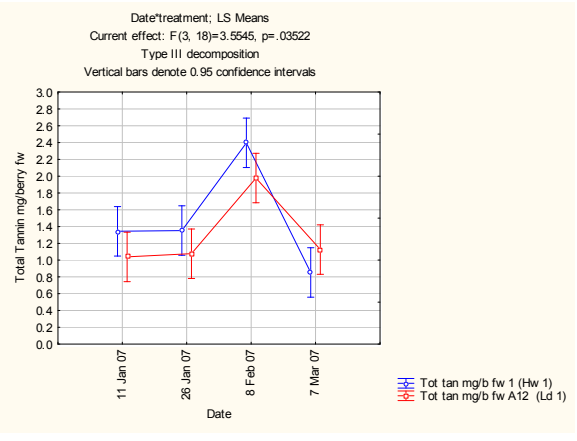


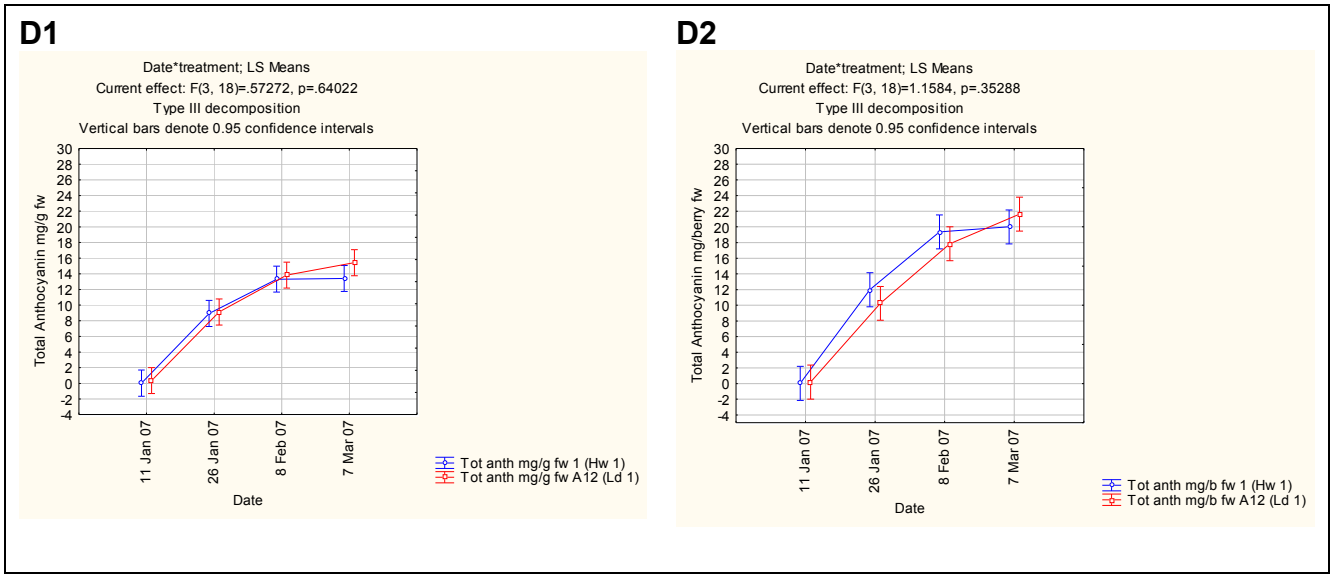
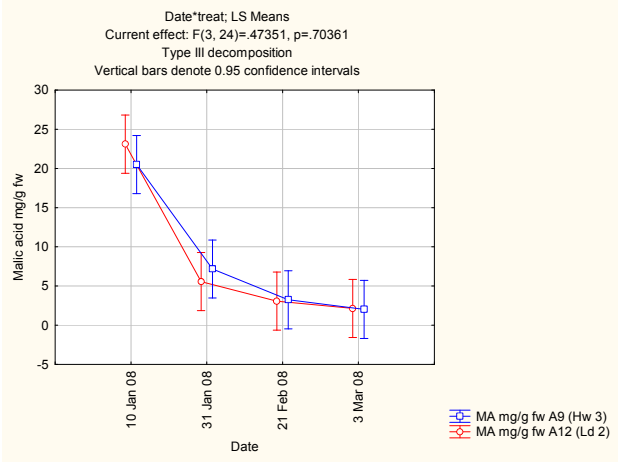
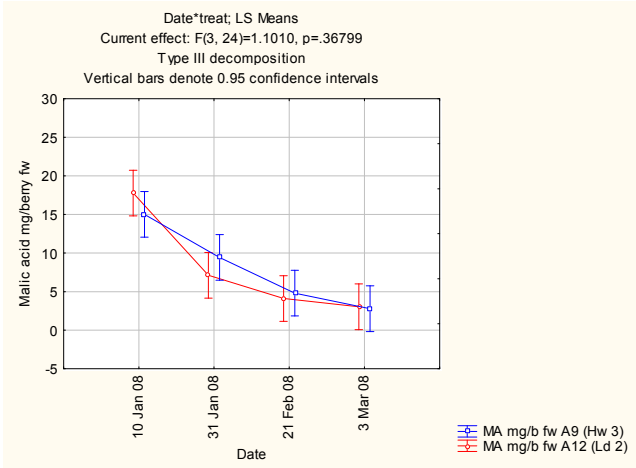


Figure 2 A. Malic acid, B. total glucose and fructose, C. total tannin and D. total anthocyanin 1. mg/g and 2. mg/berry fresh weight (fw) for two extreme plots: high vigour plot, with high soil moisture (Hw 1) and a low vigour plot with low soil water (Ld 1) during four stages (11 Jan 07 pre-veraison; 26 Jan 07 veraison; 8 Feb 07 post-veraison; 7 Mar 07 harvest) of ripening of the 2006/2007 season.

A1



A2



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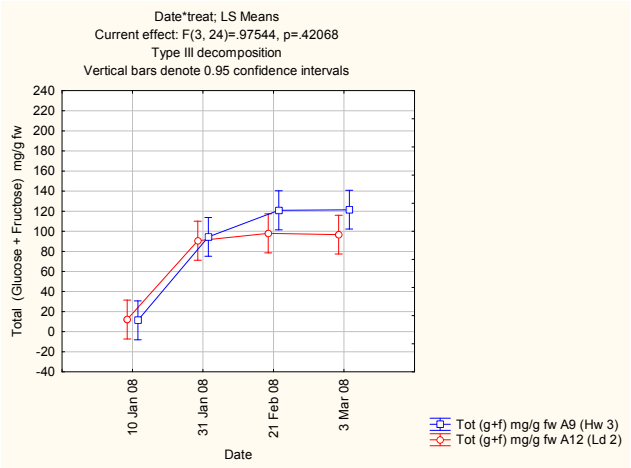

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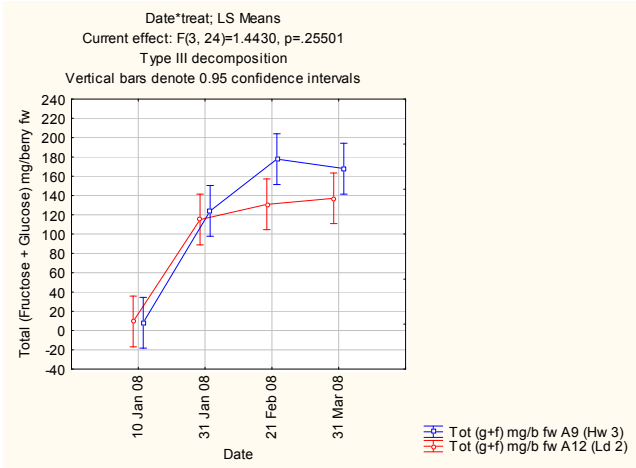
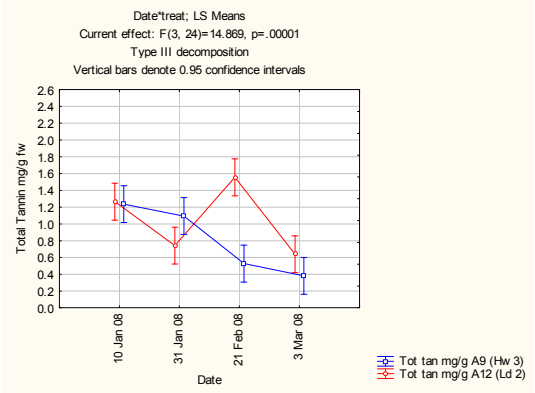


C1



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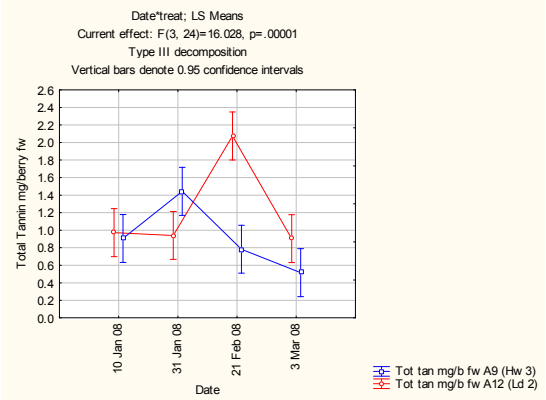

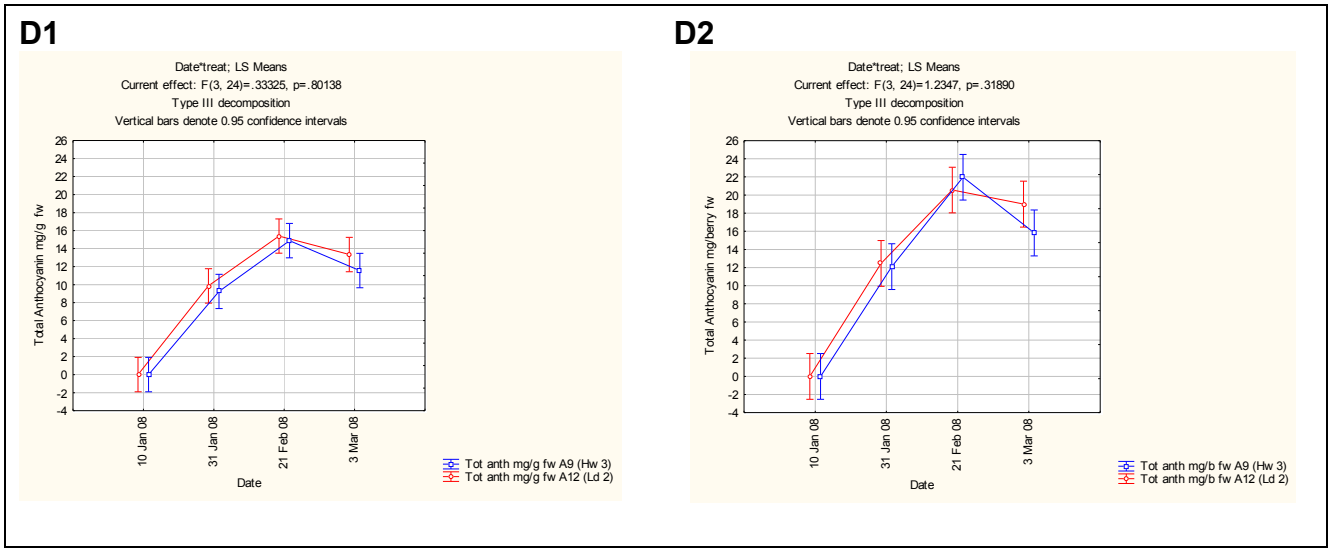

Figure 3 A. Malic acid, B. total glucose and fructose, C. total tannin, D. total anthocyanin 1. mg/g and 2. mg/berry fresh weight (fw) for two extreme plots: high vigour plot, with high soil moisture (Hw 3) and a low vigour plot with low soil water (Ld 2) during four stages (10 Jan 08 pre-veraison; 31 Jan 08 post-veraison; 21 Feb 08 post-veraison; 7 Mar 08 harvest) of ripening of the 2007/2008 season.

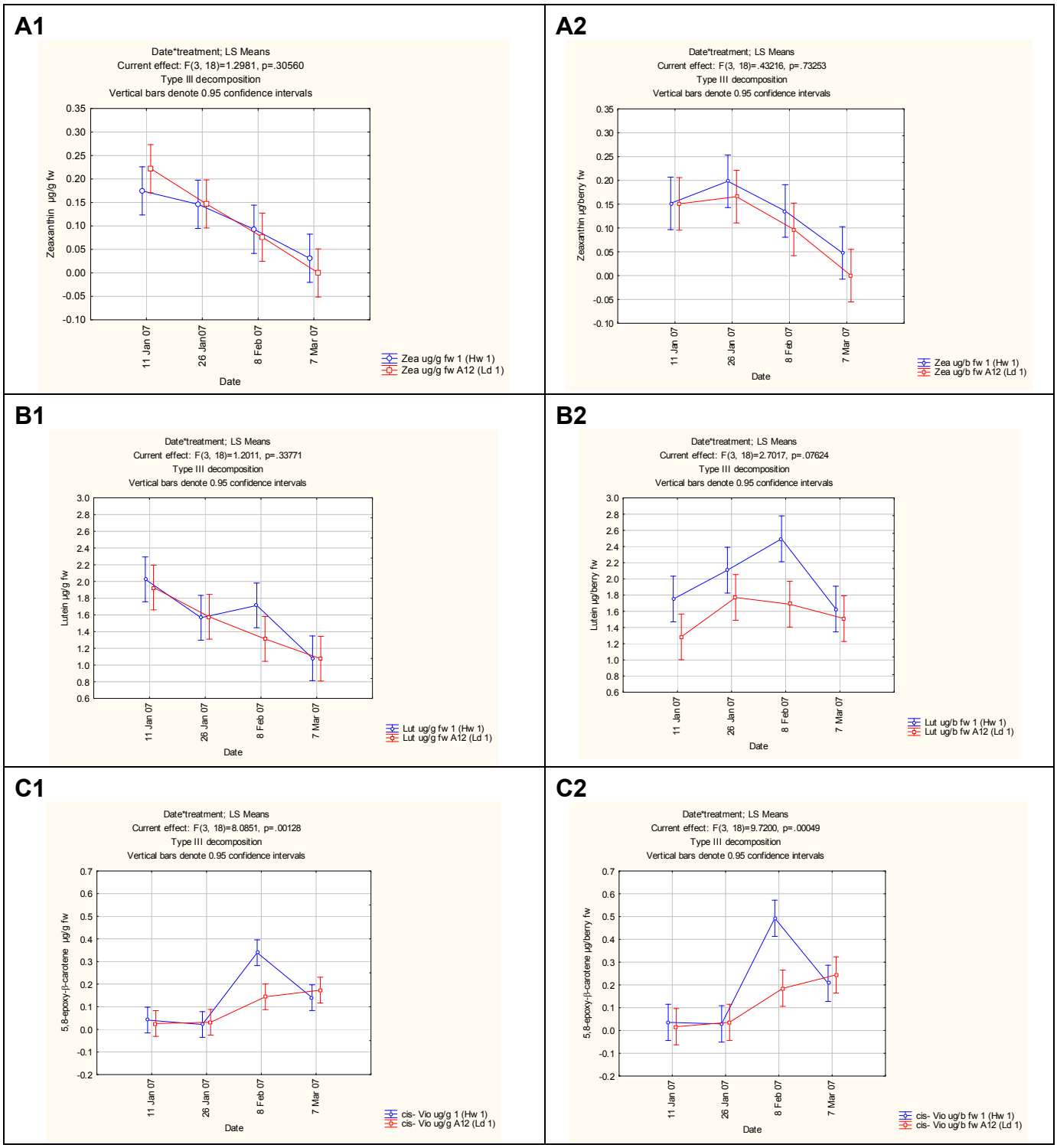

Figure 4a Individual (A to E) and (F) total carotenoid 1. µg/g and 2. µg/berry fresh weight (fw) for two extreme plots: high vigour plot, with high soil moisture (Hw 1) and a low vigour plot with low soil water (Ld 1) during four stages (11 Jan 07 pre-veraison; 26 Jan 07 veraison; 8 Feb 07; post-veraison; 7 Mar 07 harvest) of ripening of the 2006/2007 season.

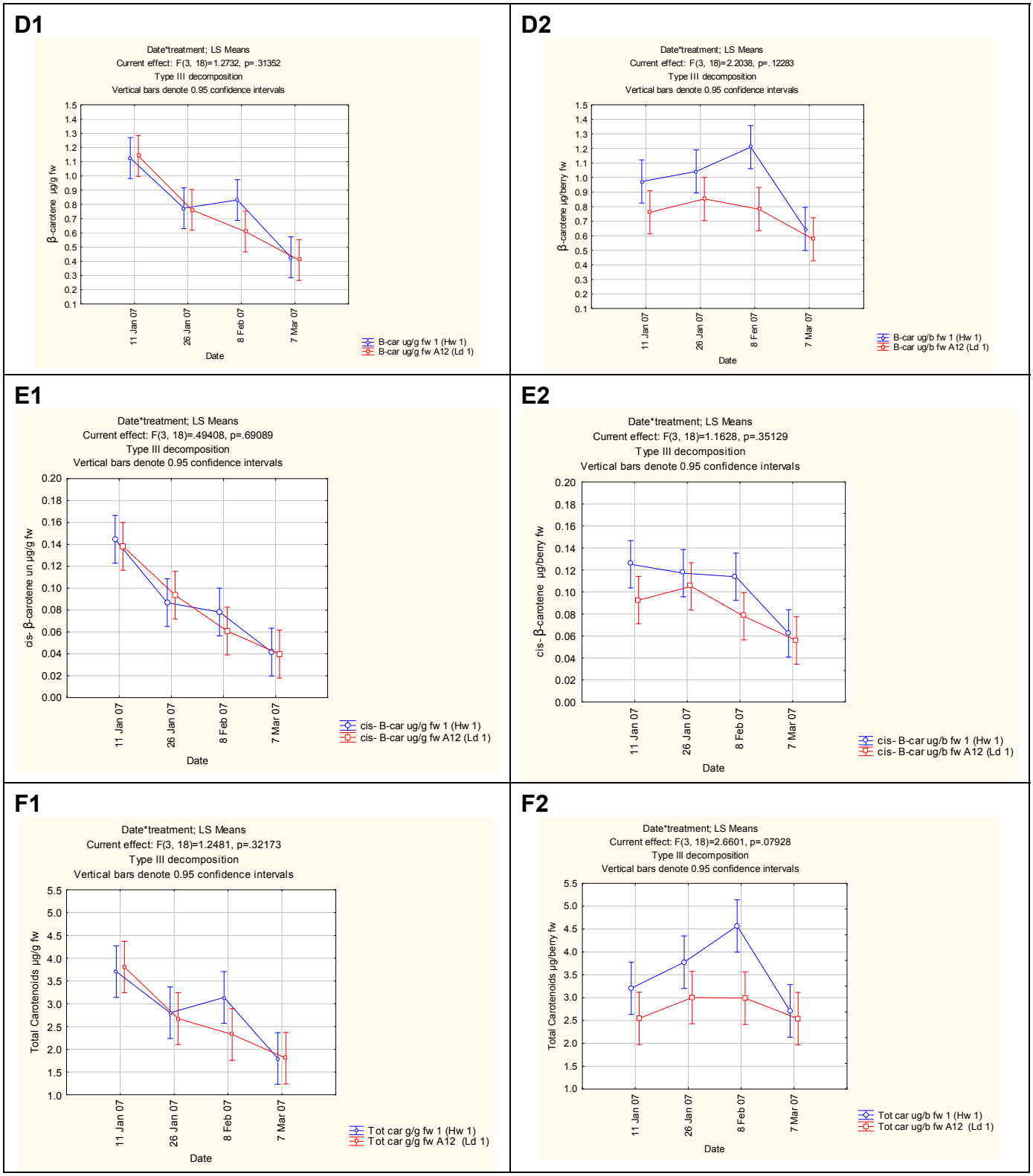

Figure 4b Individual (A to E) and total (F) carotenoid 1. µg/g and 2. µg/berry fresh weight (fw) for two extreme plots: high vigour plot, with high soil moisture (Hw 1) and a low vigour plot with low soil water (Ld 1) during four stages (11 Jan 07 pre-veraison; 26 Jan 07 veraison; 8 Feb 07; post-veraison; 7 Mar 07 harvest) of ripening of the 2006/2007 season.

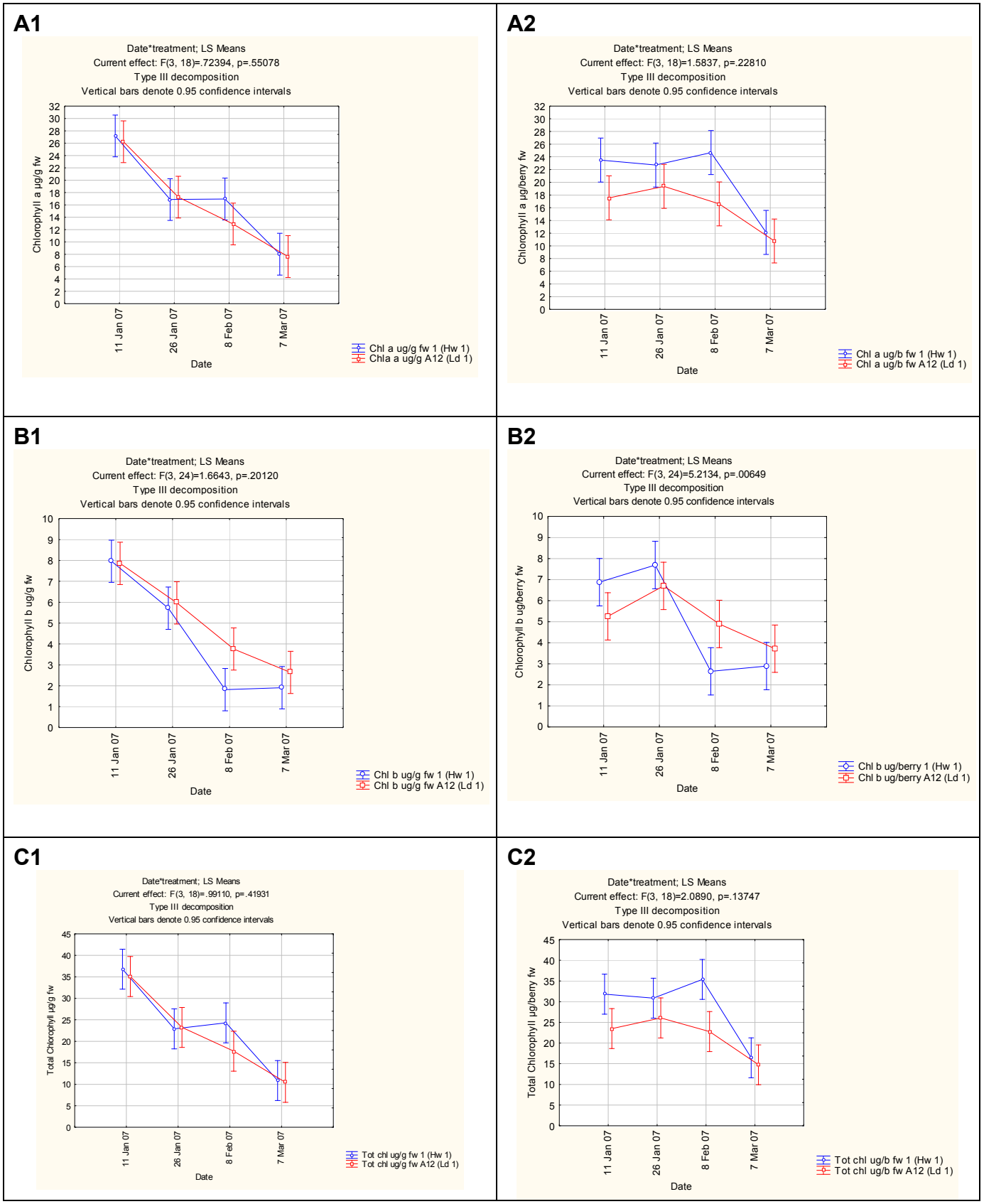

Figure 5 Individual (A and B) and C. total chlorophyll 1. µg/g and 2. µg/berry fresh weight (fw) for two extreme plots: high vigour plot, with high soil moisture (Hw 1) and a low vigour plot with low soil water (Ld 1) during four stages (11 Jan 07 pre-veraison; 26 Jan 07 veraison; 8 Feb 07; post-veraison; 7 Mar 07 harvest) of ripening of the 2006/2007 season.

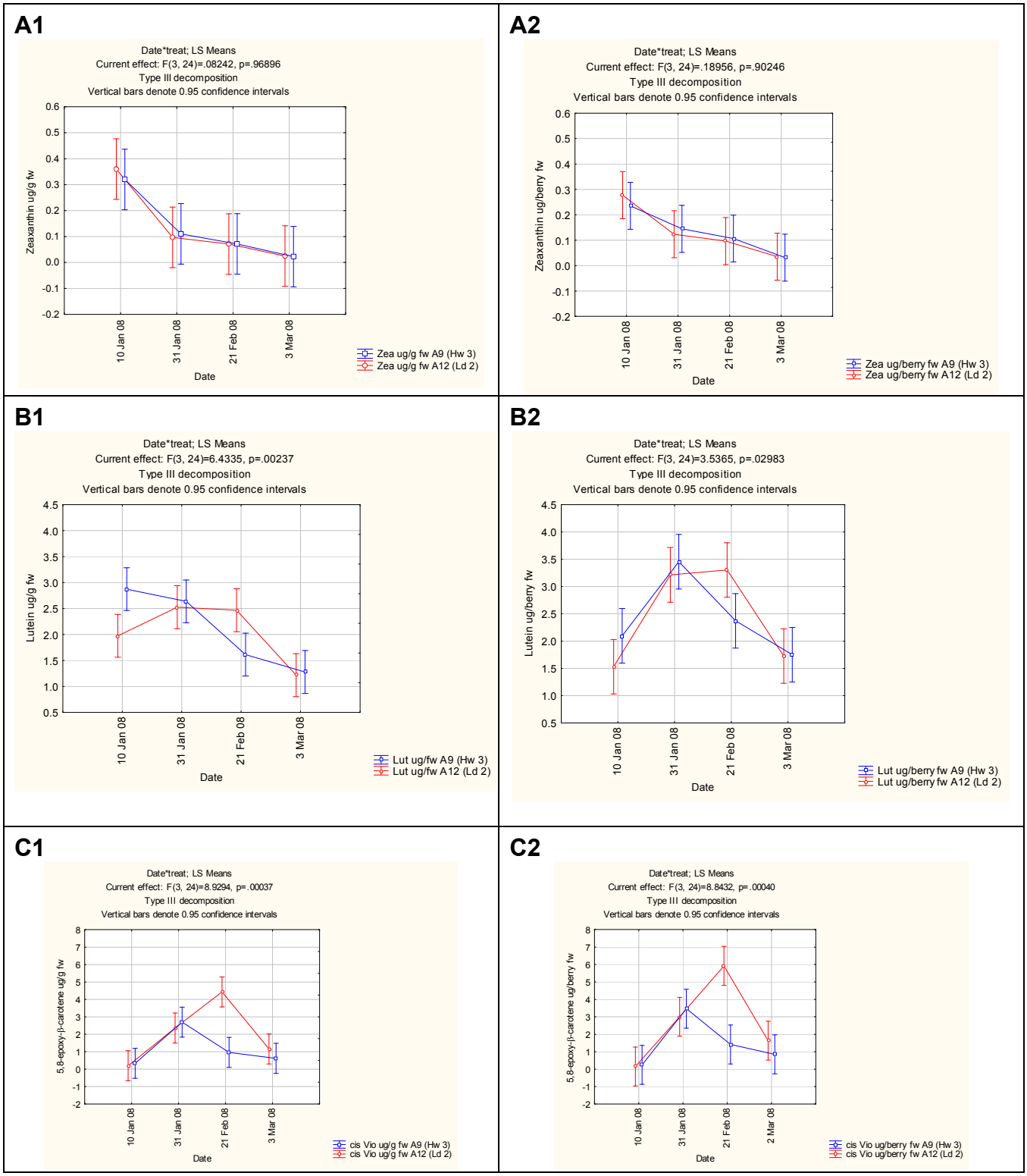

Figure 6a Individual (A to E) and (F) total carotenoid 1. µg/g and 2. µg/berry fresh weight (fw) for two extreme plots: high vigour plot, with high soil moisture (Hw 3) and a low vigour plot with low soil water (Ld 2) during four stages (10 Jan 08 pre-veraison; 31 Jan 08 post-veraison; 21 Feb 08 post-veraison; 3 Mar 07 harvest) of ripening of the 2007/2008 season.

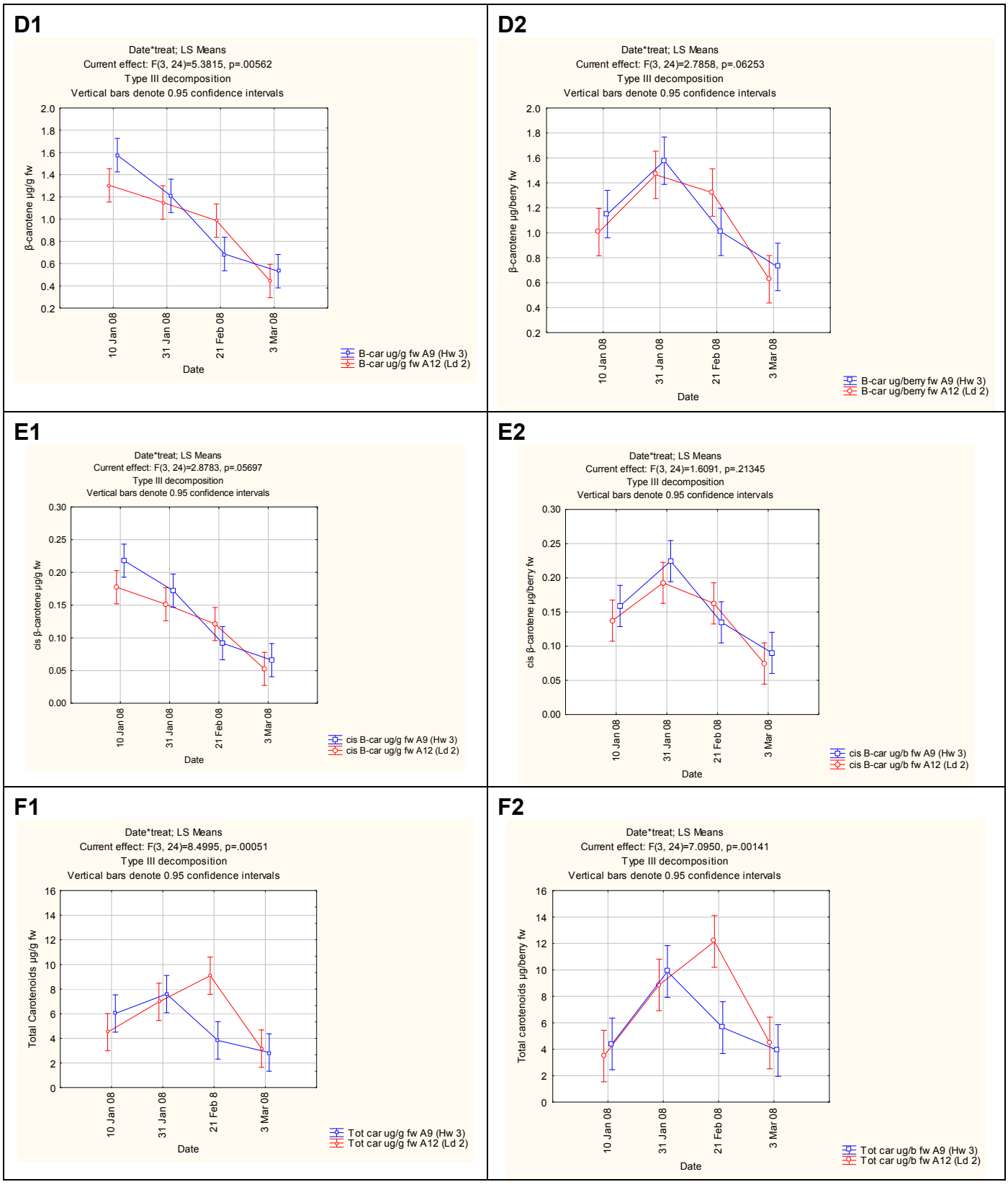

Figure 6b Individual (A to E) and total (F) carotenoid 1. µg/g and 2. µg/berry fresh weight (fw) for two extreme plots: high vigour plot, with high soil moisture (Hw 3) and a low vigour plot with low soil water (Ld 2) during four stages (10 Jan 08 pre-veraison; 31 Jan 08 post-veraison; 21 Feb 08 post-veraison; 3 Mar 07 harvest) of ripening of the 2007/2008 season.

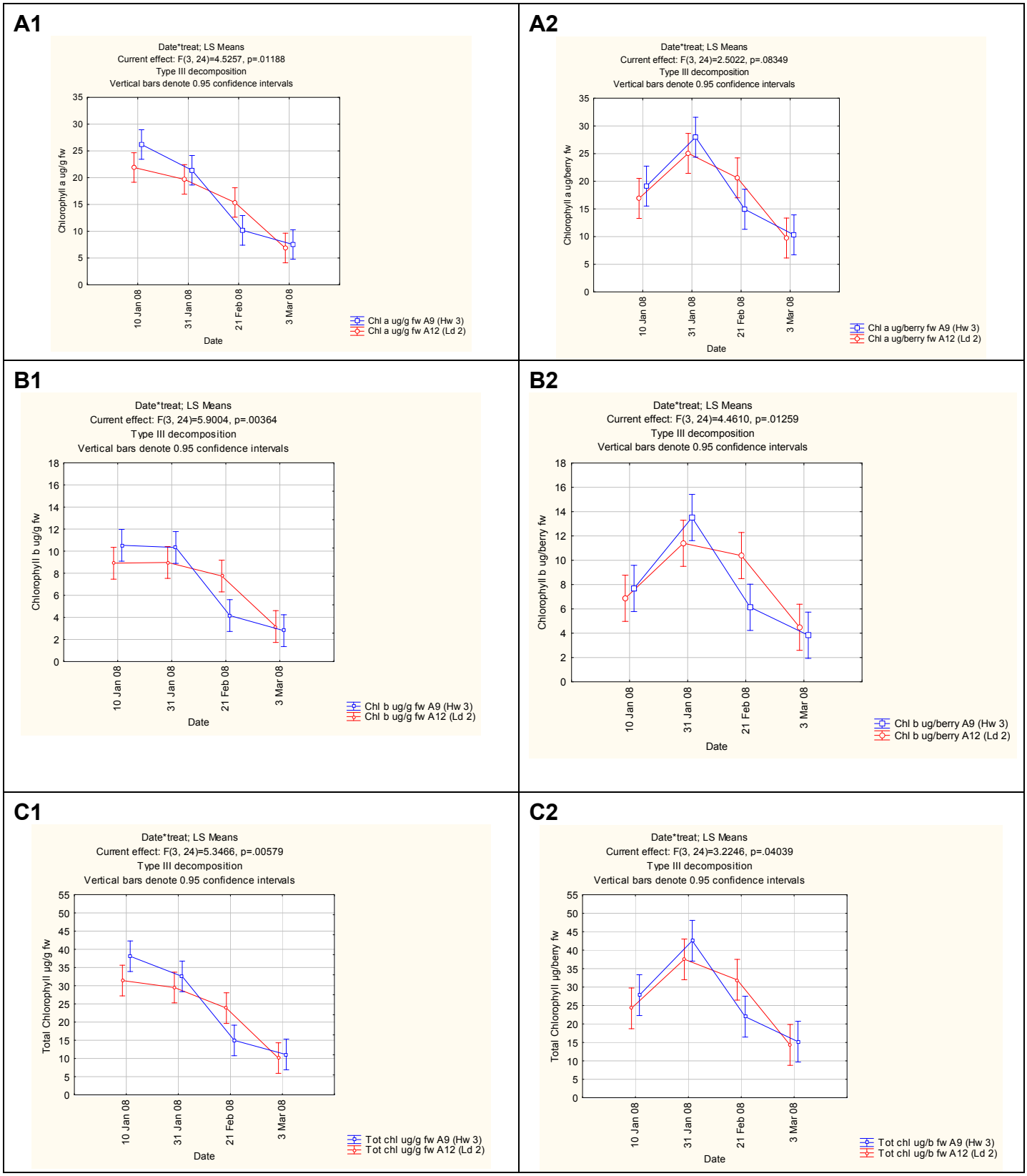

Figure 7 Individual (A and B) and total (C) chlorophyll 1. µg/g and 2. µg/berry fresh weight (fw) for two extreme plots: high vigour plot, with high soil moisture (Hw 3) and a low vigour plot with low soil water (Ld 2) during four stages (10 Jan 08 pre-veraison; 31 Jan 08 post-veraison; 21 Feb 08 post-veraison; 3 Mar 07 harvest) of ripening of the 2007/2008 season.

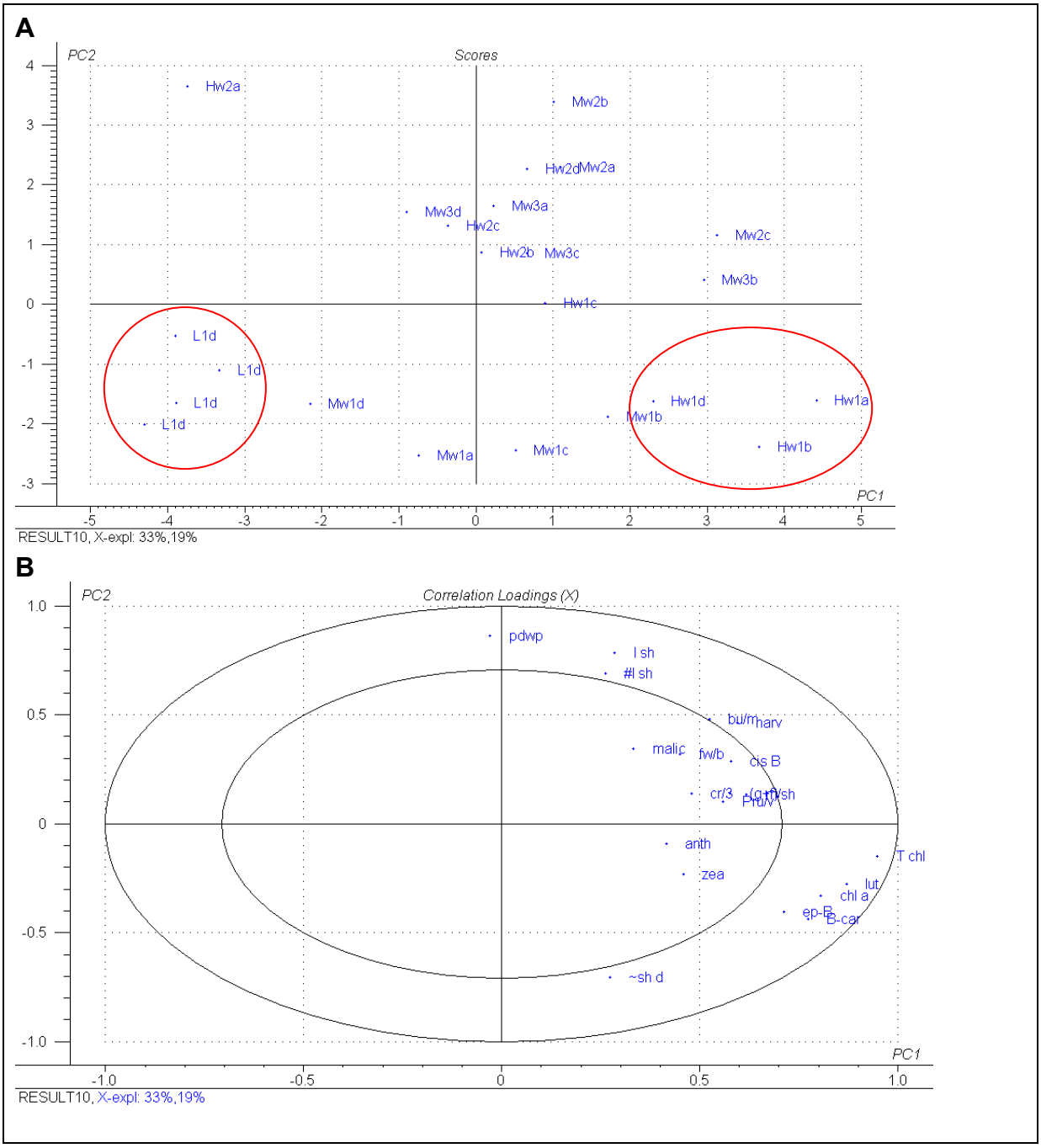

Figure 8 PCA analysis of vineyard variables, ripening parameters and carotenoids and chlorophyll content of berries post-veraison (8 Feb 07) 2006/2007 season. A. *Scores:* high vigour wet plots (Hw 1;Hw 2); medium vigour wet plots (Mw 1; Mw 2; Mw 3) and low vigour plot (Ld 1), (a; b; c and d; indicates the four replicates from sub-plots). B. *Correlation loadings (X):* average seasonal predawn plant water potential (pdwp); average seasonal soil water content (cr/3) later shoot length (l sh); pruning mass per vine (pru/v); average number of shoots per vine (# sh); number of lateral shoots (#l sh); average shoot diameter (~sh d) bunch mass (bu/m); yield per vine (harv); fresh weight per berry (fw/b) malic acid (malic); total glucose and fructose (g+f); total anthocyanin (anth) 5,8-epoxy-β-carotene (ep-B); lutein (lut); β-carotene (B-car); chlorophyll a (chl a); *cis*- β-carotene (cis-B); zeaxanthin (zea); chlorophyll b (chl b); Total carotenoids (T car); Total chlorophyll (T chl).

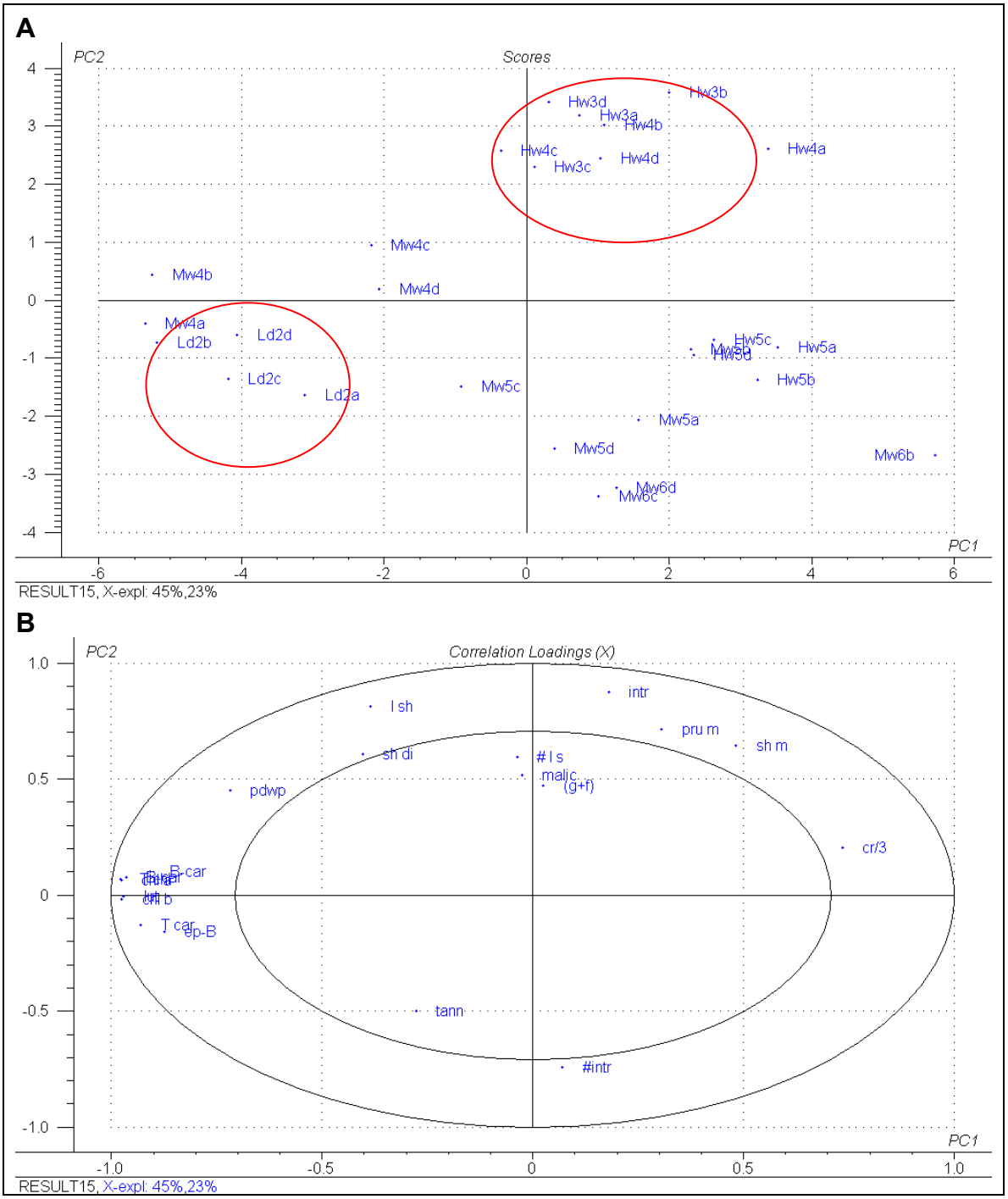

Figure 9 PCA analysis of vineyard variables, ripening parameters and carotenoids and chlorophyll content of berries post-veraison (21 Feb 08) 2007/2008 season. A. *Scores:* high vigour wet plots (Hw 3;Hw 4; Hw 5); medium vigour wet plots (Mw 4; Mw 5; Mw 6) and low vigour plot (Ld 1), (a; b; c and d; indicates the four replicates from sub-plots). B. *Correlation loadings (X):* average seasonal predawn plant water potential (pdwp); average seasonal soil water content (cr/3) lateral shoot length; internode length (intr); (l sh); pruning mass per vine (pru m); average shoot mass average (sh m); number of lateral shoots per shoot (#l sh); average shoot diameter (sh di) bunch mass (bu/m); malic acid (malic); total glucose and fructose (g+f); total anthocyanin (anth) 5,8-epoxy-β-carotene (ep-B); lutein (lut); β-carotene (B-car); chlorophyll a (chl a); *cis*- β-carotene (cis-B); zeaxanthin (zea); chlorophyll b (chl b); Total carotenoids (T car); Total chlorophyll (T chl).

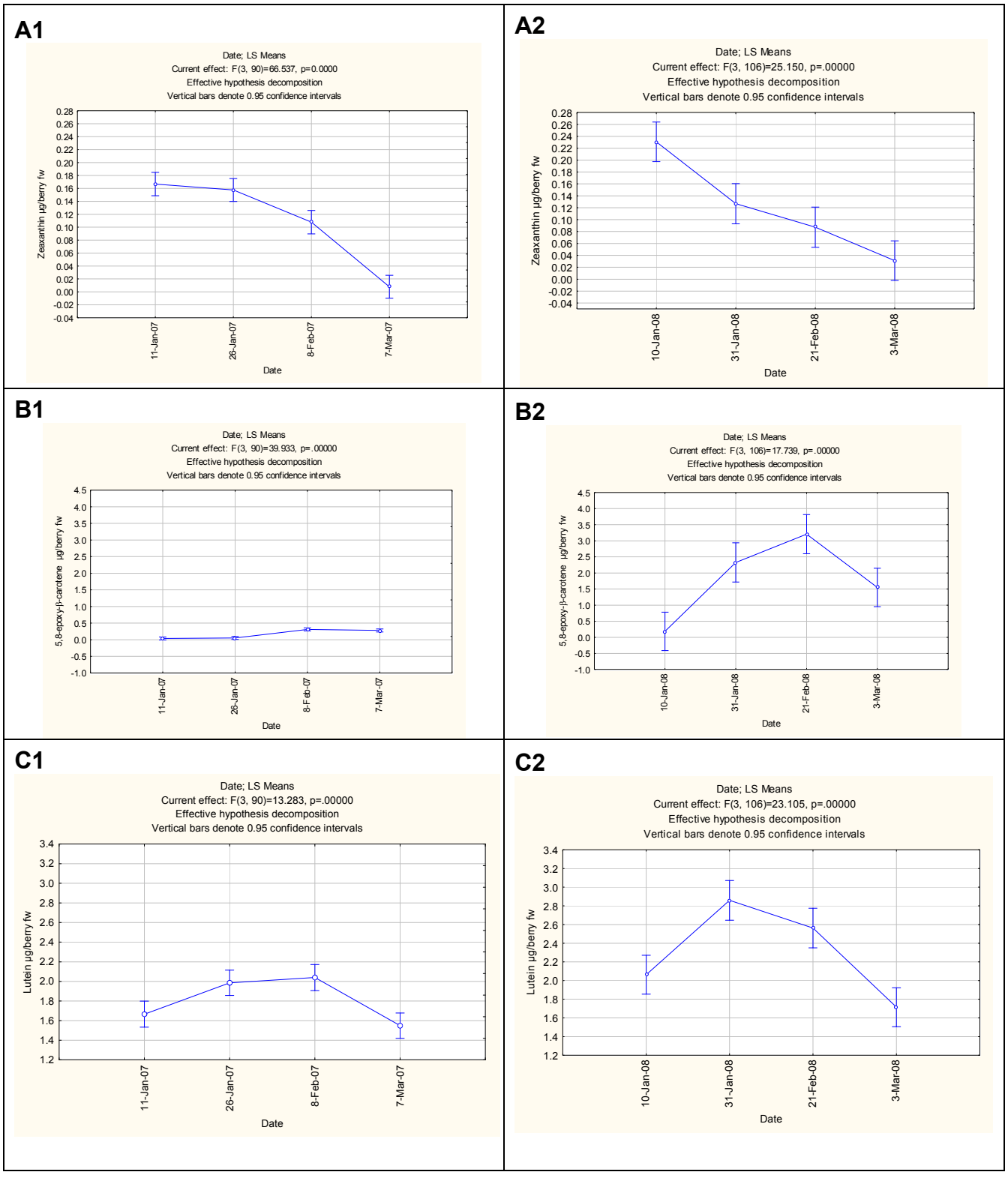

Figure 10a Individual (A to E) and total (F) carotenoid content µg/berry of Merlot berries of four ripening stages during the 1. 2006/2007 (pre-veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) and 2. 2007/2008 (pre-veraison 10 Jan 08; post- veraison 31 Jan 08; post-veraison 21 Feb 08; harvest 3 Mar 08) seasons respectively.

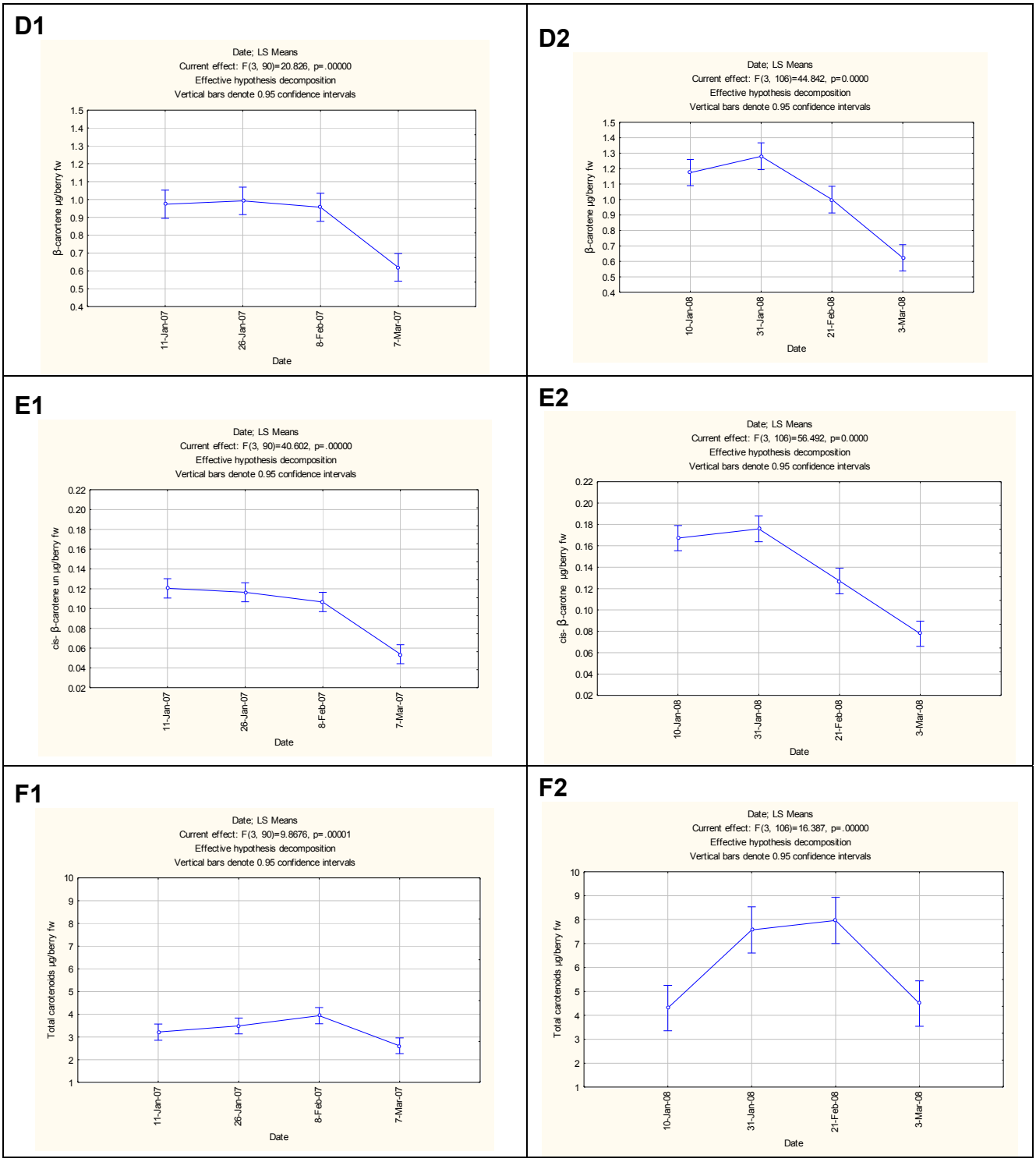

Figure 10b Individual (A to E) and total (F) carotenoid content µg/berry of Merlot berries of four ripening stages during the 1. 2006/2007 (pre-veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) and 2. 2007/2008 (pre-veraison 10 Jan 08; post- veraison 31 Jan 08; post-veraison 21 Feb 08; harvest 3 Mar 08) seasons respectively.

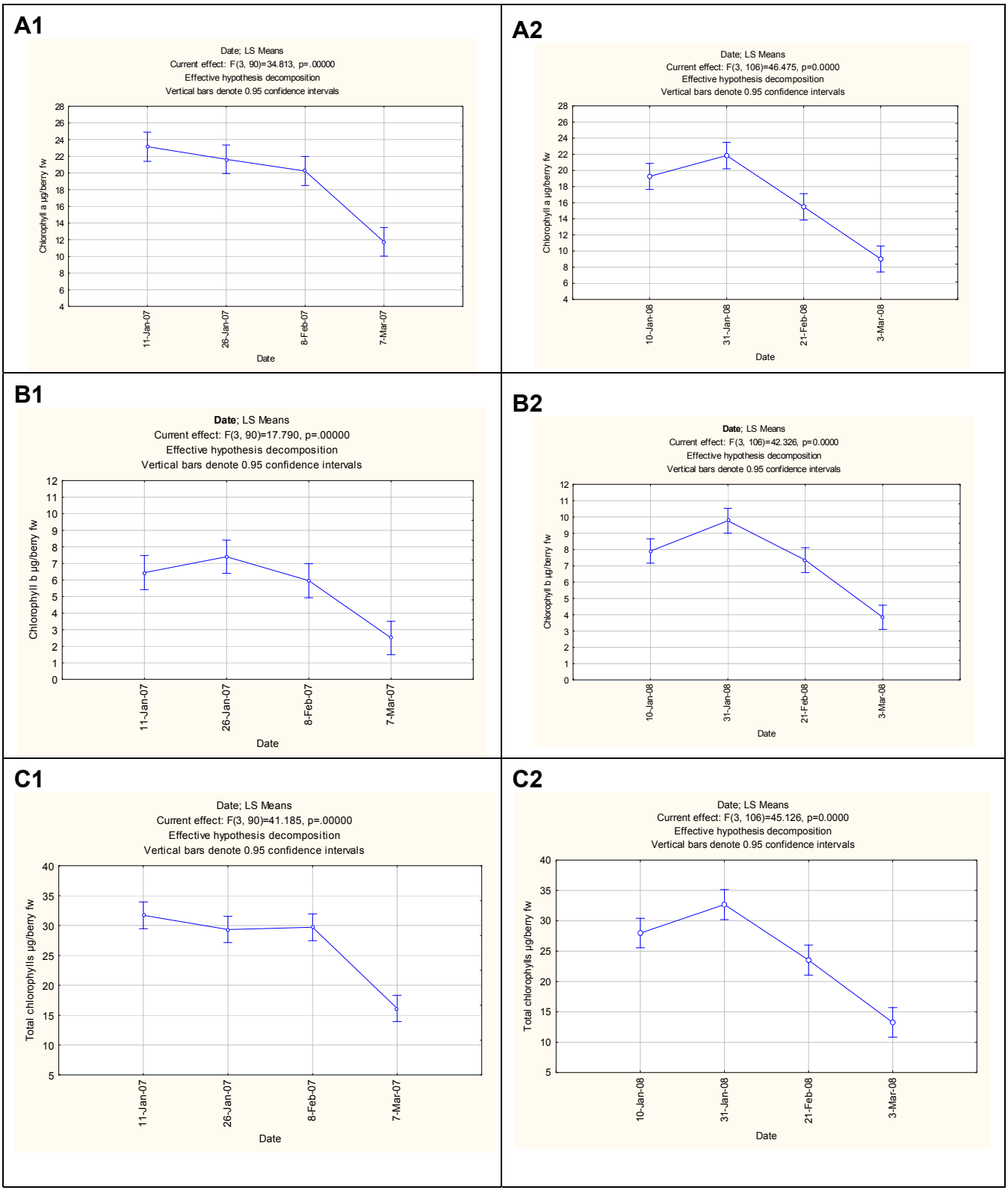

Figure 11 Individual (A and B) and total (C) chlorophyll content of Merlot berries of four ripening stages during the 1. 2006/2007 (pre-veraison 11 Jan 07; veraison 26 Jan 07; post-veraison 8 Feb 07; harvest 7 Mar 07) and 2. 2007/2008 (pre-veraison 10 Jan 08; post- veraison 31 Jan 08; post-veraison 21 Feb 08; harvest 3 Mar 08) seasons respectively.